INHERITED VARIATION OF HOST RESPONSE TO EXPERIMENTAL PERIODONTAL DISEASE IN THE MOUSE

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ABSTRACT

A mouse model for the induction of periodontal disease by the oral inoculation of live Actinomyces viscosus at concentrations of 1.6×10^6 cfu, 1.6×10^7 cfu and 1.6×10^9 cfu was first described in 1981 by Jordan et al. While many of the previous findings are confirmed, this thesis has extended the model both by examining the effects produced in eight different mouse genotypes and by increasing the number of host responses examined.

In all genotypes, bone loss determined from defleshed hemimandibles was maximal in those animals which had received inoculations of 1.6×10^7 cfu live Actinomyces viscosus. There was no apparent relationship between inoculum dose and the number of organisms recovered. This was interpreted as suggesting that periodontal destruction occurred as a result of the host response to the initial inoculum.

A consistent pattern of specific serum anti-Actinomyces viscosus immunoglobulin A emerged, with peak levels in animals which had maximum bone loss and which had beem inoculated with 1.6×10^7 cfu Actinomyces viscosus. It was postulated that the high levels of this immunoglobulin actively sequestered foreign antigen within the gingival crevice and promoted bone loss, perhaps in association with osteoclast activating factor. It was further suggested that the other two inoculum dosages induced low and high zone tolerance respectively, which could account for the low level of periodontal destruction and the low immunoglobulin response in these inoculum groups.

Cellular immunity studies including macrophage suppression tests were undertaken but the results were not detailed enough to either confirm or refute the hypotheses generated by the bone loss and immunoglobulin findings.

Polymorph studies showed a negative correlation between myeloperoxidase activity and the ratio of 1.6×10^7 cfu-induced bone loss to the control, and a positive correlation between chemiluminescence and this ratio over all genotypes. These results suggest that myeloperoxidase activity is essentially protective, whereas overall polymorph function is essentially destructive.

Histological findings were inconclusive but further investigation is already underway.

Preliminary attempts at immunisation revealed that intravenous inoculation of live Actinomyces viscosus conferred protection against bone loss normally induced by oral inoculation of 1.6×10^7 cfu.

The study has highlighted the value of immunological mutants in disclosing components of the immune system of importance in induced periodontal disease in the mouse, and has posed a number of questions that could form the basis of future work.

DECLARATION

I declare that this thesis has been composed by myself and that the work described is my own.

Angela D Gilbert

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"Where there is much desire to learn, there of necessity will be much arguing, much writing, many opinions: for opinion in good men is but knowledge in the making."

John Milton (1608-1674)

CONTENTS

CHAPTER 1 - INTRODUCTION (Contd) Page

CHAPTER 2 - MATERIALS, GENERAL METHODS AND PRELIMINARY EXPERIMENTS (Contd)

CHAPTER 3 - BONE LOSS **Page**

CHAPTER 6 - POLYMORPHS (Contd) **Page**

CHAPTER 7 - LYMPHOCYTES

CHAPTER 7 - LYMPHOCYTES (Contd) **Page**

CHAPTER 8 - IMMUNOGLOBULINS **Page**

C H A P T E R 1

INTRODUCTION

1.1 HUMAN PERIODONTAL DISEASE

1.1.1 CONTRIBUTION TO DENTAL ILL HEALTH

Inflammatory periodontal disease is one of the most widespread ailments to affect man. In 1978, the Adult Dental Health Survey reported that 82% of the adult dentate population in England, Scotland and Wales had gingivitis and that 27% had periodontitis (Todd et al, 1982). The treatment of periodontal disease has recently become a growth area within the General Dental Service (GDS) (Elderton & Eddie, 1983). In 1975 less than 1% of courses of treatment involved periodontal therapy other than routine scaling but by 1982 this had increased to 3.1% in Scotland and 7.5% in England and Wales (Dental Estimates Board Annual Report, 1976, 1983; Summary Report of the Scottish Dental Estimates Board, 1976, 1983). Only 12.1% of the total cost of the GDS went on scaling and other periodontal treatment in 1982 in England, Scotland and Wales, which compares with 51.7% on restorative treatment. Thus, in spite of the high prevalence of periodontal disease, with its possible implications for eventual tooth loss, a relatively small proportion of the resource is devoted to its prevention and treatment.

The reasons for this are many, but a 5 year longitudinal survey of 720 Scottish adults by Eddie and Davies (1984), showed that even in patients designated as having severe periodontal disease, only 25.4% were aware of having had bleeding gums in the four weeks before the start of the survey and only 7.7% were aware of having swollen, inflamed gums. Thus, because periodontal

disease patients are often symptom free, patient awareness of the disease is low. Consequently, the demand for treatment is low.

In absolute financial terms, it has been estimated that the total annual cost of treatment for the proportion of the United States' population that receives periodontal care is currently 1.5 billion dollars. In order to provide care for the more than 25 million Americans with periodontal disease who receive no treatment, the minimal cost would be over 7 billion dollars per year (United States Public Health Service Survey: quoted in Polson & Goodson, 1985). Thus, inflammatory periodontal disease has a substantial impact on public health and expenditure and is therefore a problem demanding a high priority in dental research.

1.1.2 DIAGNOSTIC METHODS

The diagnostic indicators currently used to assess periodontal status are directed towards measuring gingival inflammation, destruction of connective tissue (through periodontal pocket depth and alveolar bone loss) and crevicular fluid flow and composition.

1.1.2.1 Gingival inflammation

Recognition of the signs of inflammatory gingival disease may be important for early diagnosis (Muhlemann & Son, 1971). Indicators used have included the colour of the gingival surfaces and whether or not gingival bleeding occurred after gentle probing (Massler & Schour, 1949). As estimation of colour change is essentially subjective, it has been suggested that gingival bleeding on probing is the more sensitive and objective clinical indicator (Nowicki et al 1981; Polson & Goodson, 1985). However,

the insertion of a periodontal probe into the gingival sulcus and its subsequent movement along the tissues has a strong subjective component, as there is no strict control of the forces applied. Indeed it has been shown that a great variation in periodontal probing pressure occurs even among experienced clinicians (Hassell et al, 1973). Periodontal probes which allow control of insertion pressure have thus been developed to reduce intra- and interexaminer variability (van der Velden & de Vries, 1978; Vitek et al, 1979). The relationship between bleeding on probing and the histological characteristics of the inflamed gingival tissue has been clarified by using a standardised probe insertion force as the stimulus for gingival bleeding followed by gingival biopsy. Reactive gingivae were found to have a significantly greater percentage of cell-rich, collagen-poor connective tissue in the region of the specimen adjacent to the tooth surface (Greenstein et al, 1981).

1.1.2.2 Pocket depth :

Connective tissue destruction - soft tissue

The periodontal probe is used for measurement of both periodontal pocket depth and loss of connective tissue attachment to a root surface. While measurements are simple to perform, reproducibility is a major problem because of variations in probing force, size of probe, angle of probe insertion and the precision with which probe calibrations can be read (Proceedings of the Workshop on Quantitative Evaluation of Periodontal Diseases by Physical Measurement Techniques, 1979). Furthermore, the probe routinely has been found to penetrate the coronal level of the

junctional epithelium (Armitage et al, 1977; Robinson & Vitek, 1979; Polson et al, 1980), thus clinical pocket depths do not coincide with histological pocket depths. This disparity may be due to variables affecting gingival integrity, notably degree of inflammation (Armitage et al, 1977; Robinson & Vitek, 1979), as well as those relating to probing technique and probe diameter (Hassel et al, 1973; van der Velden & de Vries, 1978; Listgarten, 1980). A number of studies over the past decade have tried to determine the precise location of probe tip penetration into the gingival tissues (Listgarten et al, 1976; Sivertson & Burgett, 1976; Spray et al, 1978; Garnick et al, 1980). When manual probing forces were used in periodontitis the probe tip either coincided with the coronal level of connective tissue attachment (Sivertson & Burgett, 1976) or was forced apical to this within the connective tissue (Listgarten et al, 1976; Armitage et al, 1977). In order to minimise the variation due to probing forces, studies were conducted using controlled probe insertion pressures (Robinson & Vitek, 1979; Polson et al, 1980) of 25 g. In all these studies the probe tip was found to be within the junctional epithelium. In the presence of visual signs of inflammation, probe penetration was greater but the tip remained in the junctional epithelium until a force of 30 g was reached at which point penetration of the connective tissue occurred. Consequently, it appears that studies are needed to clarify the relationships between probe penetration and both clinical and histological signs of inflammation.

1.1.2.3 Bone loss

The traditional method of assessing bone loss has been from dental radiographs. Here too, the accuracy is only fair, due to variables such as angulation of X-rays and differences in processing. Although improvements have been made, there is still not enough sensitivity to detect the initial bone loss associated with periodontitis, to visualise morphology of osseous defects between the cortical plates and to obtain images of buccal and lingual bone levels (Chilton & Miller, 1977). In spite of these limitations, standardised radiographs have been shown to be valid in evaluating changes in longitudinal studies (Rosling et al, 1976; Polson & Heijl, 1978) and clinical trials (Kelly et al, 1975).

Orthopantomographs have been compared with both long-cone paralleling and short-cone bisecting techniques and, while there was agreement between techniques for approximately 50% of the teeth, orthopantomography had a tendency to underestimate minor marginal bone destruction and overestimate major marginal bone destruction (Grondahl et al, 1971). Xerography is a diagnostic X-ray imaging process which uses a xerographic copying system to read X-ray images, and has been applied to dental structures (Gratt et al, 1977; 1978; Gratt, 1979). It has been claimed that fine structures, such as bone trabeculae, and subtle density differences such as those found in gingival soft tissues, are made more visible due to a phenomenon termed edge enhancement. A comparison between conventional radiographs and dental xerographs indicated that the latter provided superior images of

the crestal alveolar bone (Gratt et al, 1980). Development of this system may thus offer an accurate, low radiation and rapid alternative to conventional intraoral radiography.

A break in the continuity of the crestal lamina dura has been described as the earliest radiographic change in periodontitis, and interpreted as a sign of active disease. It is thought that the changes are due to extension of inflammation from the interdental gingiva to the alveolar bone, resulting in a loss of bone density. The importance of density changes in the crestal lamina dura has been emphasised in early diagnosis of periodontal breakdown (Proceedings of the Workshop on Quantitative Evaluation of Periodontal Diseases by Physical Measurement Techniques, 1979). A study by Greenstein et al (1981) demonstrated that the integrity of the crestal lamina dura, evaluated on either periapical or bitewing radiographs, was not related to the presence or absence of clinically observed inflammation, bleeding on probing, periodontal pocketing, or loss of connective tissue attachment in the corresponding interdental area. These findings suggest that caution should be exercised when using integrity of crestal lamina dura as an indicator of disease activity or the need for periodontal treatment. Digitisation of radiographic images and subsequent computer subtraction has resulted in a significant improvement in diagnostic interpretation of small periodontal bone lesions (Grondahl & Grondahl, 1981).

An ^{125}I absorption technique developed by Henrikson (Henrikson, 1967; Henrikson & Julin, 1971) involves passing a 1 mm

diameter highly collimated photo beam just beneath the alveolar crest, midway between the approximal surfaces of two adjacent teeth, and comparing bone mass measurements at different time points. Initial findings indicate that the method can detect alterations in mineral density of the crestal region. However, interpreting these changes as being the result of active periodontal disease is difficult as there are several sources of error associated with reproducibility of beam location and angulation and the technique only evaluates an extremely small bone field. In addition, it is possible that such changes in bone density could be caused, at least in part, by normal bone turnover (Polson & Goodson, 1985).

1.1.2.4 Crevicular fluid flow and composition

Several studies in the last decade have confirmed earlier findings that crevicular fluid flow shows a high correlation with clinical assessment of gingivitis and/or microscopically observed inflammation (Oliver et al, 1969; Rudin et al, 1970; Wilson & McHugh, 1971; Cimasoni, 1974; Shapiro et al, 1979). Fluid measurements have therefore been considered an indirect method for measuring gingival inflammation in experimental situations. However, the interpretation of fluid flow rates from periodontal pockets is uncertain (Cimasoni, 1974).

Measurement of crevicular fluid flow has been performed by both intra- and extracrevicular techniques with the former being criticised for providing a stimulus which may itself initiate flow (Polson & Goodson, 1985). Fluid collection has involved using either calibrated microcapillary tubes or filter paper strips

placed into, or at the entrance of, the crevice for a fixed time period. In the microcapillary tube method, measurement of the fluid volume is time consuming and requires a large sample size to be considered accurate (Golub & Kleinberg, 1976). In the filter paper method, there can be variation in depth and position of strip placement, type of paper used and the techniques for visualising the fluid area on the paper (Washer, 1973). An electronic meter (Harco Periotron) has been developed which provides a direct measure of the fluid volume collected on filter paper strips. However, difficulties with instrument calibration have been reported (Taggart et al, 1980; Eid et al, 1981).

High crevicular fluid flow rates have been observed in clinically healthy sites (Muhlemann & Son, 1971) and it has been suggested that the fluid results initially from an osmotic gradient set up between plaque and tissues and later, after penetration of the basement membrane by biologically active molecules, from the development of an inflammatory exudate (Pashley, 1976; Alfano et al, 1976). These ideas may in part explain the high flow rates at healthy sites.

Attempts have been made to measure the concentration of common electrolytes in crevicular fluid but the results are inconsistent and need further clarification (Matsue, 1967; Kaslick et al, 1970; Bang et al, 1973).

Collagenase content of crevicular fluid has been examined (Golub & Kleinberg, 1976; Golub et al, 1976) and it has been suggested that tissues with chronic periodontitis produce more collagenase than do normal tissues (Robertson & Simpson, 1976).

However, the work is complex and the assays are regarded with reservation (Polson & Goodson, 1985).

1.1.2.5 Conclusions

There exists a critical need for objective diagnostic methods to enable clinicians to identify periodontal sites having active disease. At present disease activity is evaluated by periodically repeating clinical evaluations and measurements, many of which, such as probing and radiography, detect disease only when it has already occurred. Elucidation of rate of disease progression would help to identify those patients whose dentitions are at a high risk of destruction. Emphasis should be placed on early recognition of gingival disease and objective diagnosis of early periodontal lesions.

1.1.3 TYPES OF PERIODONTITIS

The forms of periodontitis observed in children and young adults have been shown to differ in many respects from those commonly seen in adults. In addition, confusion has arisen because of ongoing controversy as to both the nature of, and terminology used to describe, periodontal diseases found in children and young adults. Page and Schroeder (1982) proposed the following classification.

1.1.3.1 Prepubertal periodontitis

This form of the disease has onset during or immediately following eruption of the primary teeth, and may be either generalised or localised to particular teeth. There is gingival hyperaemia and inflammation with spontaneous haemorrhage and cauliflower-like gingival proliferation. Gingival clefts may

subsequently be formed and, with time, the periodontal tissues recede to the tooth apex. Alveolar bone and root resorption are rapid (Altman et al, 1980) and, in some cases, the primary teeth are lost by age 2-3 years (Rosenthal, 1951; Hawes, 1960). The gingival cellular infiltrate is dominated by plasma cells and lymphocytes (Rosenthal, 1951; Altman et al, 1980). Two cases of localised prepubertal periodontitis have been reported (Altman et al, 1980) in which only the free margin of gingival tissue showed acute inflammation and proliferation and in which disease progression was much slower than in its generalised counterpart. It has been suggested by Baer (1971) that periodontitis *per se* does not occur in children, except as a manifestation of some other disease state. However, cases of generalised as well as localised prepubertal periodontitis have been described in otherwise apparently normal children (Fourel, 1974; Pleasants & Nelson, 1975; Altman et al, 1980).

Prepubertal periodontitis may be followed either by completely normal periodontal status in the permanent dentition (Pleasants & Nelson, 1975) or by periodontitis associated with the permanent teeth (Rosenthal, 1951; Giansanti et al, 1973; Jorgenson et al, 1975). Disease associated with the permanent teeth may be severe enough to cause loss of many if not all permanent teeth by the mid-teens (Rosenthal, 1951; Giansanti et al, 1973). Six cases of prepubertal periodontitis have been shown to have defective neutrophils or monocytes as determined by *in vitro* chemotaxis or phagocytosis tests (Roberts & Walker, 1976; Shurin et al, 1979; Altman et al, 1980). In addition, observations indicate that

prepubertal periodontitis is more prevalent in females than in males and that it tends to run in families (Fourel, 1972; Altman et al, 1980).

1.1.3.2 Juvenile periodontitis

There is a great deal of discussion regarding the type of periodontitis occurring in teenagers and young adults. It is an uncommon condition, characterised by severe loss of attachment and destruction of alveolar bone around one or more permanent teeth in an otherwise healthy adolescent (Davies et al, 1985). Baer et al (1963b) and Baer (1971) found that the amounts of microbial deposits present were not consistent with the degree of periodontal destruction. The disease was first accurately described by Wannenmacher (1938) who noted that lesions were confined to incisor and first molar regions only. Deep periodontal pockets could be present while the gingival tissue appeared healthy. However, the disease may also be more generalised (Davies et al, 1985). Baer (1971) considered that localised and generalised forms were different manifestations of the same disease process. Age of onset is circumpubertal. In localised juvenile periodontitis prevalence in females is higher than in males, with a female to male ratio varying from 2 to 3.5:1 (Benjamin & Baer, 1967; Manson & Lehner, 1974; Melnick et al, 1975). A familial pattern has been identified in a number of studies (Butler, 1969; Jorgenson et al, 1975; Melnick et al, 1975) and X-linked inheritance has been suggested (Melnick et al, 1975; Fretwell et al, 1982), but both sexes are affected and an autosomal recessive pattern of inheritance seems more likely (Saxen, 1980).

Studies of HLA antigens in juvenile periodontitis have been equivocal but have provided some evidence of a negative association with HLA-A2 and of positive associations with HLA-A9 and HLA-B15 (Saxen & Koskimies, 1984; Tiwari & Terasaki, 1985) suggesting the possibility of an immunogenetic basis for the disease.

The lesions progress with extreme rapidity immediately after onset, and destruction appears to slow with time (Baer & Socransky, 1979). Recent data indicate that, while there is evidence that localised disease may become generalised with time (Hormand & Frandsen, 1979), the two forms may be distinct, as summarised below.

Localised form

In localised juvenile periodontitis, Actinobacillus actinomycetemcomitans, a Gram-negative facultative anaerobic rod, has been found in large numbers and is thought to play a dominant role in the disease process (Slots, 1976; Slots & Rosling, 1983; Haffajee et al, 1984). Resolution of disease follows its elimination from subgingival plaque and re-establishment of the organism is associated with disease recurrence. Patients have elevated serum immunoglobulin specific to the organism and high levels of specific antibody in crevicular fluid and gingival tissue (Chen et al, 1983; Haffajee et al, 1984). Following successful treatment, the levels of antibody to this species decrease significantly in both serum and crevicular fluid. The organism has been found within gingival connective tissue of affected individuals (Saglie et al, 1982).

Between 75% and 80% of patients with localised juvenile periodontitis have depressed neutrophil chemotaxis (Clark et al, 1977; Genco et al, 1980b; Van Dyke et al, 1980). This defect has been shown to be intrinsic in that the neutrophils have a reduced number of cell surface receptors for chemotactic factors (Van Dyke et al, 1981; 1983). There is evidence that the chemotactic defect is familial and that it persists into adulthood, even after treatment of the disease (Van Dyke et al, 1980).

Generalised form (rapidly progressive periodontitis)

In this form of juvenile periodontitis, the first molars and incisors are frequently among the teeth involved, but periodontal destruction appears often to extend to other parts of the dentition. Many patients may not present until the third or fourth decade of life, making information concerning the onset and progression of disease difficult to obtain. Evidence for progression from the localised to the generalised form comes from studies by Hormand and Frandsen (1979), Baer and Socransky (1979) and Vandesteen et al (1981). However, Burmeister et al (1984) showed that 39% of their patients with juvenile periodontitis still had only first molar/incisor involvement when aged 21-30 years, implying that progression to the generalised form may not be inevitable. Prevalence in females is higher than in males but, in contrast to the localised form, no evidence of familial occurrence has yet been found.

Preliminary studies of thirty patients with generalised juvenile periodontitis by Davies et al (1985) have failed to detect a consistent defect of neutrophil function. Page et al

(1983a; 1984) however reported a range of neutrophil chemotactic activity in such patients, as well as abnormalities of monocyte chemotaxis.

Data concerning the microflora associated with generalised juvenile periodontitis are sparse, but certain Gram-negative, anaerobic bacteria, notably Actinobacillus species and Bacteroides gingivalis, have been implicated (Ranney et al, 1981; Slots, 1982; Page et al, 1983a). These organisms have considerable pathogenic potential and could provoke the severe tissue destruction found in this condition (Slots & Genco, 1984). Page and co-workers (1983a) believed generalised juvenile periodontitis to be a distinct and entirely separate entity from the localised form and were the first to use the term rapidly progressive periodontitis.

1.1.3.3 Adult periodontitis

The most common type of periodontitis is a chronic disease termed 'Adult Periodontitis' by Page and Schroeder (1982). They described it as being "characterised by an age range of 35 years and older in which the presence of microbial deposits is commensurate with the amount of periodontal destruction seen. Bone loss can be either generalised or localised, horizontal or vertical but not of the distinct pattern seen in juvenile periodontitis. There is no evidence of rapid progression, no predisposing systemic diseases and no blood leucocyte abnormalities. The pocket flora is characterised by a mixture of gram negative microorganisms containing, among others Bacteroides melaninogenicus ss intermedius, Eikenella corrodens, vibrios and spirochaetes, but without a significant population of B. gingivalis or Actinobacillus actinomycetemcomitans".

Adult periodontitis responds well to plaque removal and root curettage and adjuncts such as antibiotic therapy are not needed. Fibrosis and other manifestations of chronic long standing inflammation may be present, but in many cases the gingival tissues have not retreated apically as alveolar destruction worsened. Preliminary data (Hirshfeld & Wasserman, 1978; Becker et al, 1979) suggest that relatively quiescent adult periodontitis may convert, at one or more sites, to a form of rapidly progressive disease and that rapidly progressive disease may revert spontaneously to the more slowly progressive adult form. There is some evidence to suggest that HLA-A2, HLA-A10 or HLA-B5 may confer a degree of resistance to adult periodontitis (Goteiner & Goldman, 1974; Amer et al, 1985; Tiwari & Terasaki, 1985).

1.1.3.4 Acute periodontitis

For the sake of completeness, it should be noted that there are two main forms of acute periodontitis, pericoronitis and periodontal abscess (Higgins et al, 1985). Pericoronitis, as the name suggests, involves inflammation of the tissues around the crown of a tooth and is most commonly associated with partially erupted third molars. A periodontal abscess may occur as an acute exacerbation of pre-existing chronic inflammatory disease or may arise de novo in association with particulate matter forced into the gum.

1.1.3.5 Other types of periodontitis

Another type of periodontitis is that observed following repeated long-term episodes of acute necrotizing ulcerative gingivitis, characterised by deep craters in the interdental bone,

either localised or throughout the mouth (Page & Schroeder, 1982). The disease undergoes periods of exacerbation during which greyish pseudomembranes composed of bacteria and necrotic tissue form in the affected regions and the breath becomes fetid. Periodontitis of this type has not been well studied.

1.1.4 PERIODONTAL PATHOGENS

The concept that periodontal diseases are bacterial in origin is supported by experiments in germ-free animals (Irving et al, 1975; Listgarten et al, 1978a), responses to both mechanical treatment and chemotherapy (Listgarten et al, 1978b; Slots et al, 1979), histological evidence of bacterial invasion of the tissues (Saglie et al, 1982; Slots et al, 1983), therapeutic efficacy of plaque control (Axelsson & Lindhe, 1978; 1981) and experimental gingivitis studies in man (Loe et al, 1965, Theilade et al, 1966). The situation has been summed up by Page & Schroeder (1982): "Organisms present in microbial plaque in the region of the gingival sulcus, or gingival or periodontal pocket, or substances derived from them constitute the primary and possibly the only extrinsic aetiological agents, participating in the aetiology of inflammatory gingival and periodontal disease." It is thus essential to know something of the organisation, pathogenic potential and composition of microbial plaque, especially subgingival, which both initiates and promotes the disease process.

1.1.4.1 Organisation of subgingival plaque

Subgingival plaque is highly structured and organised yet, in any given pocket, there are significant differences in microbial

composition between one location and another and significant changes in composition with time (Soames & Davies, 1974; Listgarten et al, 1975). The build up of plaque on subgingival surfaces has been studied by using removable epoxy crowns (Listgarten et al, 1975). The surfaces were first colonised by Gram-positive cocci which, on multiplication, formed columns. Within one week, the coccoid plaque became covered by filamentous organisms and within three weeks, these latter forms both invaded and replaced the cocci. At the advancing front of the pocket, or on the soft tissue side thereof, the flora was made up of plaque-related, freely floating, unattached spirochaetes, small unidentified cocci, Gram-negative rods, many of which were motile, and unusual microbial aggregates referred to as 'corn-cobs'. Bacteria have also been identified within the epithelium and connective tissues at the apical terminus of advanced pockets as well as at surfaces of and within alveolar bone (Michel, 1969; Frank, 1980; Manor et al, 1984).

1.1.4.2 Pathogenic mechanisms

The possible means by which bacteria and the substances they release cause periodontal damage may be summarised as follows (Page & Schroeder, 1982):

(a) Rapid growth and apical extension of the area invaded by microorganisms requires space which is created by displacement through physical force (exudation) or enzymatic activity of the junctional epithelium from the tooth surface (Schroeder & Attström, 1980).

(b) Some of the bacteria produce leucotoxins which hamper neutrophil defence (Taichman & Wilton, 1980; Baehni et al, 1980).

(c) Bacterial components may interact with various host cells and systems to activate acute inflammation and possibly immuno-pathological processes which cause pathological tissue alteration (Page & Schroeder, 1973, 1976; Smith et al, 1980).

(d) Some bacterial products can directly cause bone resorption (Daly et al, 1980; Nowotny et al, 1982).

1.1.4.3 Microbial floras associated with periodontal health and disease

In one of the earliest investigations in this field, Hemmens & Harrison (1942) found no major differences between the periodontal flora of normal and diseased human subjects. On the other hand, Rosebury et al (1950) showed that certain organisms such as spirochaetes, vibrios and fusiform bacilli were present in the scrapings from periodontally diseased teeth in far greater numbers than in those from periodontally normal teeth.

These latter observations were not supported by the studies of Socransky et al (1963) or Gibbons et al (1963) who found no significant differences of composition between floras of diseased and healthy sites, although there was a difference in the total amount of microbial deposit present. Examination of the ratio of non-motile to motile organisms found in diseased as compared to healthy sites was, however, more fruitful. In clinically normal or recently treated pockets this ratio was found to be 40:1 (Listgarten & Hellden, 1978; Darwish et al, 1978; Rosenberg et al, 1981). In contrast, the ratio at diseased sites was shown to be

approximately 1:1 (Listgarten & Hellden, 1978). Numerous studies have attempted to associate different floras with different periodontal disease states, resulting in a large collection of data, much of which is contradictory. However, the following general points appear to be widely accepted.

Healthy Sites

Newman et al (1976) studied the flora of healthy pockets in patients with localised juvenile periodontitis. The sites had a predominantly Gram-positive and aerobic flora comprising mostly Streptococcus sanguis, S. mitis, Staphylococcus, Propionibacterium acnes and large numbers of Gram-positive filamentous rods of the Actinomyces type. Studies by Slots (1979) and Tanner et al (1979) have shown that Streptococci and facultative species of Actinomyces, especially A. viscosus and A. naeslundi, along with Rothia dentocariosa, account for up to 85% of the total cultivable flora at clinically normal sites. Saccharolytic Bacteroides species and Capnocytophaga have also been found (Spiegel et al, 1979).

Advanced periodontitis

In general, the flora at diseased sites with little associated gingival inflammation is primarily Gram-negative and anaerobic (Listgarten & Hellden, 1978). However, Tanner et al (1979) found that B. melaninogenicus ss intermedius, pleomorphic Bacteroides and Eikenella corrodens predominated. With greater degrees of gingival inflammation, Bacteroides gingivalis and Fusobacterium nucleatum accounted for up to 75% of the cultivable flora (Slots, 1979) while Tanner et al (1979) showed the presence

of anaerobic vibrios and pleomorphic Bacteroides also. Lai et al (1981) and Holt et al (1981) have described the presence of previously unidentified Gram-negative bacteria in advanced periodontitis lesions.

Juvenile periodontitis

The idea that specific microorganisms may be associated with particular clinical entities has recently been supported by serum immunoglobulin studies of patients with generalised juvenile periodontitis. Such individuals showed raised serum titres to B. gingivalis (Mouton et al, 1981; Ebersole et al, 1982). Patients with localised juvenile periodontitis, however, demonstrated low titres to B. gingivalis but raised titres to Actinobacillus actinomycetemcomitans (Murray & Genco, 1980; Listgarten et al, 1981; Ebersole et al, 1982; Ranney et al, 1982). Indeed, individuals with localised juvenile periodontitis have elevated total serum immunoglobulin levels (Lehner et al, 1975; Kaslick et al, 1980; Chen et al, 1983) and high levels of specific anti A. actinomycetemcomitans immunoglobulins in serum, saliva and gingival crevicular fluid (Genco et al, 1980a; Murray & Genco, 1980; Listgarten et al, 1981; Ebersole et al, 1982; Ranney et al, 1982). Such elevated titres in patients with juvenile periodontitis were shown to decrease after successful treatment (Genco et al, 1980a).

Slots & Rosling (1983) showed that, unlike some other periodontopathogens, A. actinomycetemcomitans could not be eliminated from pockets by mechanical debridement alone. According to Zambon (1985), this is probably due to the organism's

ability to invade gingival connective tissues, evidence for which has been provided by the electron microscopic studies of Saglie et al (1981a,b) and Gillett & Johnson (1982). Specific identification of the organism was performed both by immunoperoxidase techniques (Saglie et al, 1982a,b) and indirect-immunofluorescent staining with species specific antisera (Christersson et al, 1983). Thus, Slots et al (1983) showed 1 g per day oral tetracycline administered for three weeks to be effective in eliminating A. actinomycetemcomitans from periodontal sites. Combined conventional periodontal therapy and systemic antibiotics resulted in decreased gingival inflammation, gains in clinical attachment and regeneration of angular osseous defects in studies by Lindhe (1982) and Slots & Rosling (1983).

Much recent work has concentrated on A. actinomycetemcomitans and its virulence factors which are capable of inhibiting host defence mechanisms. These are:

(a) Leucotoxin, a heat stable protease sensitive product (Tsai et al, 1984) which lyses human polymorphonuclear leucocytes (Baehni et al, 1979; Tsai et al, 1979) and human monocytes (Taichman et al, 1980) by causing cell membrane perturbations. At healthy sites only 7% of the small number of A. actinomycetemcomitans present are leucotoxin producing while producers constitute 43-75% in diseased subjects (Baehni et al, 1981; Zambon et al, 1983). Resultant polymorph damage releases hydrolytic enzymes to exacerbate local tissue damage (Zambon, 1985).

(b) Polymorphonuclear leucocyte chemotaxis inhibitor, which impairs these cells' response to chemotactic agents (Van Dyke et

al, 1982) and may exacerbate the systemic neutrophil chemotactic defect found in some localised juvenile periodontitis patients.

(c) Another soluble, heat labile factor, which suppresses lymphocyte activation to mitogens and antigens (Shenker et al, 1982b), though its significance is uncertain.

(d) Catalase from the organism, which can inhibit oxidative bacterial killing by degrading hydrogen peroxide of host immune cells (Slots, 1982).

(e) Lipopolysaccharide from the organism, which participates in the Schwartzman reaction, macrophage activation and platelet aggregation (Kiley & Holt, 1980) and is a potent mitogen (Chen et al, 1981), causing bone resorption in organ culture.

(f) Collagenase produced by most strains (Robertson et al, 1982; Rozanis & Slots, 1982; Rozanis et al, 1983), which may combine with that from lysed neutrophils to produce tissue damage.

(g) Another factor which inhibits growth and proliferation of human fibroblasts (Shenker et al, 1982a; Stevens & Hammond, 1982) which, in conjunction with the collagenase, may not only destroy tissue but also inhibit repair (Stevens & Hammond, 1982).

(h) Epitheliotoxin (Birkedal-Hansen et al, 1982; Kamen, 1983), which may assist the organism in sulcular epithelial penetration and connective tissue access.

Thus this organism is periodontopathic from many different points of view. Some or all of these factors may also be produced by other bacteria which have not, as yet, been as extensively tested.

1.1.4.4 Effects of sampling

As pointed out by Greenstein & Polson (1985) the act of site microbiological sampling can introduce variability into studies and clinical diagnoses. Mousques et al (1980) demonstrated that repeated sampling of sites can affect their microbial composition and Listgarten & Lewis (1967) showed that returning to sample identical locations is difficult as the bacterial composition varies in different regions of the same lesion. Furthermore, collection of specimens at different pocket depths introduces another variable, since there is evidence that the percentage of spirochaetes and motile rods may be pocket-depth related (Armitage et al, 1982; Savitt & Socransky, 1984). However, other workers have shown that deep pockets do not necessarily contain increased percentages of these morphotypes (Evian et al, 1982; Greenwell & Bissada, 1984).

1.1.4.5 Conclusions

The importance of bacteria in the pathogenesis of periodontal disease is beyond doubt. However, in view of the difficulties in merely sampling sites, far less culturing, counting and identifying the organisms recovered, it is not surprising that a very large number of conflicting views as to the details of microbial involvement have been put forward. The flora is enormously complex and many of the bacteria are extremely fastidious in their growth requirements. Nevertheless, further understanding of the organisms and pathogenic mechanisms involved in the different types of disease is desirable for rational prevention and therapy.

1.1.5 HOST DEFENCE SYSTEM

The components of the mammalian host defence system in the periodontium include the intact epithelial barrier; vessels of the microcirculation with their neighbouring mast cells; factors from the blood plasma, including specific and non-specific antibodies, opsonins and the complement, clotting and plasminogen proteins; the kinin system; and the peripheral blood leucoctyes including the neutrophils, monocytes and lymphocytes. The various components operate in concert; their activities are interrelated and highly interdependent, and there are myriads of control mechanisms, most of which are not well understood. The host defence system component cells can also exert direct effects on the resident cells such as fibroblasts and bone cells, thus eliciting direct changes (Irving et al, 1979; Garant, 1979; Narayanan et al, 1980).

It appears that activation of any component of the host defence system, be it the immune response, acute inflammation, the complement cascade, the phagocytic leucocytes, or various combinations of these, creates the potential for a great deal of tissue damage as well as for potentially life-saving protection (Page & Shroeder, 1982). Normally the damage created can be readily repaired and the price paid, in terms of tissue damage, is small relative to the protection provided.

The factors determining whether or not tissue damage will occur and, if so, how much, and the duration of the destructive process are only poorly understood. It is known that chronic inflammatory diseases, including periodontitis, continue so long

as the driving force remains present, and all tend to resolve when the driving force, microorganisms, in the case of periodontitis, is inactivated or removed. In addition to the nature of the driving force, which components of the host defence system are called into play, the exact manner in which they are activated, and the magnitude of their participation, determines the type and extent of the tissue damage. This is further complicated as it can vary from one individual to another, from one disease state to another and even from one stage of a given disease to another.

The roles of the polymorphonuclear neutrophilic granulocytes and both cellular and humoral immune systems will be examined in detail in subsequent chapters. It is, however, possible to say that the host defence system at large, with its double-edged role of protection and destruction, is perhaps the most important element in periodontal disease initiation and progression. The complex interrelationships of the different components make separate study difficult, but perhaps attempting this is the only way to advance our understanding of periodontal disease.

1.1.6 CONCLUDING REMARKS

A number of types of investigation of human periodontal disease have been performed, including diagnostic, epidemiological, therapeutic and both cellular and humoral immunity studies. Most of these have provided data on small numbers of patients in discrete populations, yet frequently the findings have been deemed to apply to the human population at large.

The most important and least soluble problem in using man to

study periodontal diseases stems from the many sources of variation both between and within populations. Examples include host genotype, oral flora, diet, concurrent therapy, standard of oral hygiene and level of patient cooperation during treatment. Furthermore, witholding treatment in order, for example, to monitor disease progression would be unethical in man.

In experimental animals, it is possible both to manipulate the different sources of variation and to observe the progress of untreated disease. In addition, large enough samples to provide unambiguous results are easily obtained. Thus, animal models in periodontal disease research, where so many basic questions with regard to host response, pathogenesis, epidemiology and therapy remain unanswered, can be a powerful way of providing clues about the human disease that would be difficult or impossible to disclose in man.

1.2 PERIODONTAL DISEASE IN EXPERIMENTAL ANIMALS

Pathological changes in the periodontal tissues of a variety of experimental animals have been studied both to understand certain aspects of disease aetiology and to test therapies that have some promise in the control of disease. Naturally occurring and induced disease have both been investigated. Species used have included larger mammals such as dogs, sheep, cattle and several non-human primates but, most commonly, rodents have been studied. Although the gross anatomy of the dentition varies greatly from one species to another, the structure and organisation of the periodontal tissues are generally similar. As a consequence, with but minor exceptions, the essential features of periodontitis are common to all mammals that have been investigated.

1.2.1 SIZE AND TIME

Animal size is extremely important not only with regard to costs of animal acquisition, maintenance, experimental design and tissue processing, but also in understanding the pattern and progression of the disease and interpreting experimental results.

Bacterial colonies and the neutrophil granulocytes they attract occupy space. In large experimental animals such as dogs and chimpanzees, the dimensions of the dentition fall within a range similar to that for humans, but in other experimental animals the teeth, alveolar bone and soft tissues are much smaller. In rodents, where there are three molars in each quadrant, tooth size ranges from that found in the pocket mouse to

that of the hamster. However, in all these animals the interdental regions are very small. Thus, the interval between approximal root surfaces of adjacent molars, although containing the same structural elements as found in humans, organised in a similar manner, measures only a fraction of a millimeter (Page & Schroeder, 1982). Under these conditions, certain questions become very important. What type and quantity of dental plaque and how many cell layers can be accommodated? What is the range of effectiveness of the bacteria and the substances they release? What is the radius or range of tissue involvement relative to the tissue volume available?

Size is also relevant for histological processing where, particularly for small animals, preparations with minimum distortion are required. Many classical histological procedures fall short of such stringent demands (Page & Schroeder, 1982). Time is also an important consideration in the individual history of periodontitis in different animals. Pathological processes, which in man and other long-lived animals may take years or decades to develop, must necessarily develop much more quickly in rodents such as mice with their short life span of sometimes less than one year. Sulcular hair impaction which occurs to varying degrees may be a precipitating factor (Baer & Lieberman, 1959, 1960).

1.2.2 MICE AS EXPERIMENTAL ANIMALS

Mice have a number of advantages over other experimental animals. They are easy to procure and, provided they are acquired

from a reputable source, should be free from infection and infestation. Many genotypes, even relatively unusual mutants, can be purchased in Great Britain so eliminating the need for quarantine.

The small size of the animals allows many of them to be housed in identical conditions, thus, in quantitative experiments, it is easy to study numbers large enough for unambiguous statistical analysis. Also, when larger numbers of animals are used, losses due to accident, disease or death are not as serious as in small samples. Mice are easy to handle and are suited to a wide variety of experimental procedures, such as germ-free housing and rearing, administration of special diets and both microbiological and therapeutic experiments. They also have a short generation time, making breeding experiments much quicker than in other animals. This also means that a few young pairs will quickly produce a thriving colony, so eliminating the need for recurrent acquisition. Cost, of course, in these days of stringent financial constraint, is a prime consideration in setting up experiments and mice are valuable in that, apart from the rarer genetic mutants, they are relatively cheap to buy, transport, house and feed. More importantly, mice are regarded as being sufficiently similar to man in relation to the anatomy of the periodontium that they may be considered to show a disease process analogous to that found in man.

Nevertheless, early investigators such as Mitchell (1954), Costich (1955) and Konig & Muhlemann (1959) noticed that the subepithelial inflammatory reaction seen in human diseased

periodontal tissue was usually absent. This is perhaps because the crevicular epithelium is keratinised in rodents but not in humans. Thilander (1961) reported that the keratinised epithelium in rats blocked the passage of trypan blue and may therefore block irritating or toxic materials from bacterial plaque. Trypan blue penetrated the tissue and entered the crevice primarily at the epithelial attachment, which was therefore considered to be the site in rodents where materials passed through the epithelial barrier. It has also been suggested by Socransky (1970) that the rodent oral flora is simpler than that of man. This could be due to the relatively simple and constant experimental diets consumed by rodents or to physiological differences between species controlling the implantation of bacteria. A simplified flora may be considered an advantage when devising pathogen-free models but it may be a limitation in terms of the total gingival ecology as seen in humans.

A number of investigators (Fitzgerald et al, 1960; Jordan et al, 1965; Gustafson, 1969) have described the phenomenon of hair impaction in the interdental spaces of rodent molars. This is a condition to which germ-free animals seem especially prone. In conventional animals, such impaction may add to the tissue irritation originating from plaque microflora and should therefore be taken into account as a possible cause of localised periodontal destruction.

However, the balance of advantages and disadvantages seems to be that, provided the investigator is aware of the differences between the mouse model and man, mice have a most important contribution to make in the investigation of periodontal disease.

1.2.3 PHYSIOLOGICAL CHANGES IN MICE

Mice show a complex series of physiological alterations involving the molar regions. Cohn (1957), reporting on RAP-albino mice, stated that "as soon as functional occlusion is attained" (M_1 at 24 days, M_2 at 25 days and M_3 at 35 days) "and all through the life of the tooth, the crown is being worn down with relative rapidity because of the enamel free areas on the cusps. To compensate for the occlusal wear, there is a gradual deposition of cellular cementum at the apical end of each tooth which keeps the teeth in occlusion. The formation of this cellular cementum is so pronounced in the mature molar, that there is a distinct hypercementosis at each root tip." Gilmore & Glickman (1959) used the same strain of mouse and showed that continuous bucco-occlusal molar eruption occurred after a functional occlusion had been attained and that the periodontal surface of the buccal plate underwent resorption while the periosteal surface showed apposition. The lingual plate showed the opposite changes. Again, apical cement deposition was noted. The distance between the alveolar crest and the amelocemental junction (ACJ) in mice therefore increases with age for non-pathological reasons. Other authors working around the same time did not appear to appreciate these normal changes (Baer & Bernick, 1957; Baer & Newton, 1959; 1960), although Baer & Lieberman (1960) and Baer et al, (1963a) showed that murine occlusal wear per unit time was dependant on the abrasiveness of the diet. The histological specimens appearing in the papers of both Baer & Bernick (1957) and Baer & Lieberman (1959) showed apical cellular cementum deposition.

1.2.4 PERIODONTAL DISEASE IN MICE

1.2.4.1 Spontaneous periodontal disease in the wild

Spontaneously occurring periodontitis has been reported by Sheppe (1965) for two deer mouse (Peromyscus) populations near Vancouver, Canada. Advanced disease with both maxillary and mandibular molar tooth loss was demonstrated in one species living at higher elevations. The disease was endemic and confined to a small geographical area. Other animals of the same species in British Colombia and Washington State, USA were not affected. Sheppe believed it to have an infectious origin, perhaps with inherited susceptibility. This Peromyscus oreas species appears to be the only one in which spontaneous periodontitis is known to occur in mice in the wild. However, similar disease patterns do occur spontaneously or can be experimentally induced in highly inbred strains of laboratory mice (see below).

1.2.4.2 Importance of age

Baer & White (1961) determined the minimum age of disease development in STR/N mice as between 30 and 50 days of age or approximately with the onset of sexual maturity. They also stated that the lesions increased in severity with age. It is probable that this "bone loss" was a reflection of normal physiological processes. Rateitschak & Reimers (1969) studied STR/N mice aged 48 - 83 days on one of three different diets for up to 450 days. Histological and ACJ-alveolar bone crest measurements revealed that periodontitis had not developed in 90 day old animals or even in those 165 days old. However the data confirmed previous observations (Gilmore & Glickman, 1959) that

the distance between ACJ and alveolar bone crest, while increasing with age, was always around twice as great on the lingual (palatal) aspects of the molars as on the buccal, and this was consistent with physiological change. Only by 365 - 450 days was periodontitis apparent and even then only moderate bone loss with irregular shallow pocketing was found.

1.2.4.3 Variation in susceptibility between strains

Histology was the means by which Baer & Bernick (1957) first described periodontitis in 10 - 24 month old C57BR/dcJN mice. They noted that changes occurred regularly in the maxilla but rarely in the mandible and that these involved the development, by 12 months, of crater-type pockets which, along with degradation of bone septa both interdentally and in furcation areas, increased in number and severity with age. Baer & Lieberman (1959; 1960) examined three strains at 16 months of age (DBA/2JN, C57L/HeN, STR/N) and a further 6 strains at age 12-24 months (SWR/N, B/HeN, BRSUNT/N, C57BL/6JN, A/HeN, A/LN). The jaws were defleshed in papain and bone loss scored 0-4 according to the number of quarters of roots exposed. The maximum bone loss around any tooth was taken as the score for that tooth. Strains STR/N and BRSUNT/N developed lesions related to massive hair impaction. SWR/N, BL/HeN, C57BL/6JN showed only moderate non-specific periodontitis regularly and moderate hair impaction. The remaining two strains (DBA/2JN, C57L/HeN) appeared to be relatively disease resistant. It was also noted that, in all susceptible strains, the disease was more severe in the maxilla than in the mandible and more severe in females than males. The differences in susceptibility

were thought to be genetic but an experiment involving cross-breeding and backcrossing of susceptible STR/N and resistant DBA/2JN mice did not provide evidence to support this hypothesis (Baer et al, 1961).

Doykos et al (1967) studied the mouse mutant grey lethal (gl) and showed that the homozygote had retardation of growth, general inability to resorb bone and no tooth eruption as root formation is halted after crown eruption. In some animals apparently typical periodontitis with deep interdental craters, bone loss and abundant microbial plaque was observed at 10 weeks of age (Cohen et al, 1969). Sheehan et al, (1972) concluded that the grey lethal mutant had a high genetic susceptibility to periodontal disease.

BNL (Brookhaven National Laboratory, USA) mice aged 5-78 weeks were studied by Tonna (1972). Histological examination of both gingival and periapical regions showed that inflammatory infiltrate, which both developed more frequently and increased in intensity with age, occurred at both sites. However, there was no associated bone loss, and the inflammatory infiltrate remained always within the gingival connective tissues. It was therefore suggested that the BNL strain is resistant to periodontitis.

Spontaneous leukaemia in various mouse strains does not seem to predispose to periodontitis. Leukaemic infiltrates have been observed in pulp tissue and periodontal membrane spaces adjacent to bone marrow spaces, but no periodontal damage developed in either ARK-leukaemic or CFWw mice (Carranza et al 1965; Brown et al, 1969; Flanagan et al, 1970).

1.2.4.4 Sublethal X-irradiation

Sublethal X-irradiation up to 9500 rad, of Swiss-Webster mice (Shapiro et al, 1960; Greulich & Ershoff, 1961) and Swiss and C57BL mice (Burstone, 1950), resulted in severely disturbed tooth development and rampant periodontitis with total loss of alveolar bone, deep periodontal pocket formation and root exposure both at interdental and interradicular sites in older animals. The large crater-like pockets were packed with bacterial deposits attached at least in part to root surfaces. A latent period of several months preceded the onset of periodontitis, which eventually came to affect all animals surviving 100 - 300 days after the first X-ray exposure. Ultimately, tooth exfoliation occurred leaving a socket containing a large mass of bacterial plaque. Such events did not occur in non-irradiated controls (Greulich & Ershoff, 1961). These results were interpreted as implying that bone marrow damage and the resulting effects on phagocytic cells were very important in disease susceptibility. In addition, Greulich & Ershoff (1961) suggested that irradiation may have altered the oral bacterial flora in some way, thus affecting disease experience.

1.2.4.5 Histological features

Most investigators have described the inflammatory periodontal lesion only superficially but they agree that, both within the junctional epithelium or pocket epithelium and in connective tissue subjacent or lateral to these epithelia, leucocytes, mostly neutrophilic granulocytes, tend to accumulate (Gilmore & Glickman, 1959; Greulich & Ehrshoff, 1961; Tonna, 1972;

Messer, 1980). In older animals, a slight infiltration of lymphocytes in a subepithelial position has frequently been observed (Baer & Bernick, 1957; Rateitshak & Reimers, 1969; Tonna, 1972). The type of periodontal disease seen in various strains of mice is characterised by the irregular occurrence, chiefly in the maxillary molar region, of wide interdental and interradicular craters filled with bacterial masses, hair and cellular debris. In spite of ulcerations seen in the epithelium lining the crater-like pockets (Baer & Bernick, 1957; Shklar & Person, 1975) true abscess formation has not been seen, even in severe lesions developing after X-irradiation (Greulich & Ehrshoff, 1961).

1.2.5 BACTERIAL INVOLVEMENT IN MURINE PERIODONTAL DISEASE

1.2.5.1 Bone loss in germ-free mice

Germ-free white Swiss mice 39 days to 6 months and 12 - 30 months of age were examined by Baer & Newton (1959; 1960) and Baer et al (1964) together with conventional mice of the same strain. The results showed that the 'bone loss' for germ-free and conventional mice was essentially the same in both age ranges. Messer (1980) however, pointed out that Swiss-Webster mice, from which Baer and colleagues' germ-free colony was derived, were essentially periodontal disease resistant. The changes described were again consistent with normal physiological change yet Baer & Newton (1959; 1960) interpreted the data as evidence of disease.

By defining periodontal disease as the combination of alveolar bone loss and apical proliferation of the epithelial attachment, they concluded that disease occurred in germ-free animals without the presence of inflammation and that "living bacteria are not the primary etiologic factors in the type of periodontal disease which occurs in germ-free mice".

1.2.5.2 Implantation of bacteria into the mouths of germ-free mice

Gibbons et al (1964) successfully implanted human oral bacteria, including a strain of Bacteroides melaninogenicus, into the oral cavities of germ-free mice and Gibbons & Socransky (1966) demonstrated, using a scoring method developed for rats (Gupta & Shaw, 1956b) that gnotobiotic mice 110 - 145 days old orally inoculated with human gingival bacteria lost more bone than did germ-free controls. Thus the inoculated bacteria presumably induced pathological damage that was superimposed on normal physiological change.

1.2.5.3 The effect of chlorhexidine

Daily oral treatment of conventional male STR/N mice with 0.2% chlorhexidine, an antibacterial agent used in the management of human periodontitis, resulted in a mean increase in the ACJ to alveolar crest distance (on the lingual aspect of each molar root) of 0.08 mm compared to 0.14 mm in untreated control mice (Messer & Douglas, 1980). This finding provides further support for bacterial involvement in pathological periodontal change in the mouse.

1.2.5.4 Induction of bone loss by *Actinomyces viscosus*

Early observations by Keyes (1946) revealed a condition in hamsters characterised by gingival accumulations of plaque around the molar teeth, resulting in soft tissue inflammation and alveolar bone loss. Mitchell & Johnson (1956) demonstrated the inhibitory effect of penicillin on this condition, implying bacterial involvement. The infectious nature of the disease was confirmed by demonstrating its transmissibility (Keyes & Jordan, 1964). In particular, while albino hamsters were not sufferers of periodontal disease they could become so if plaque was transferred to them from the mouths of affected golden and cream hamsters (Jordan & Keyes, 1964). By using this model, it was possible to show that the disease was induced by specific filament-forming organisms isolated from infected hamsters. Cultural characteristics and morphology (Howell, 1963) as well as biochemical properties (Howell & Jordan, 1963) indicated the similarity of this organism to certain actinomycetes. Because inclusion in the genus *Actinomyces* was not recommended at that time, the organism was named *Odontomyces viscosus* (Howell et al, 1965). Later, Georg et al (1969) proposed that the genus be amended to include catalase-positive strains and the organism was renamed *Actinomyces viscosus*.

Development of the rat model as an experimental system for studying periodontal disease followed a pattern similar to that traced for the hamster. Early observations on spontaneous gingival disease noted masses of debris and plaque-like accumulations in the gingival crevice (Konig & Muhlemann, 1959;

Stewart & Burnett, 1958; Thilander, 1961). The rice rat, because of particular susceptibility to periodontal disease, has proved well suited to this type of study (Gupta & Shaw, 1956a). Again, administration of antibiotics decreased the severity of periodontal disease (Gupta et al, 1957) thereby suggesting a bacterial aetiology. As in the hamster, periodontal disease in the rice rat was shown to be transmissible (Dick & Shaw, 1966) and later the infectious agent proved to be a Gram-positive, rod-forming organism (Dick et al, 1968). This description fits with that of Actinomyces viscosus although no name was given to the organism. Similar organisms have been found in the cervical plaque obtained from humans, and various strains have been isolated from human dental calculus (Gerenczer & Slack, 1969). In addition, Jordan (1971) stated "in our laboratory we have routinely isolated catalase positive, filament forming bacteria from human material. Many strains are morphologically and biochemically similar to the animal strains." It was the finding of specific bacteria in animal models that prompted a search for these organisms in humans. Actinomyces viscosus was consequently regarded as a possible aetiological agent in human periodontal disease by Jordan et al in 1972. Several other reports have demonstrated the association of A. viscosus with dental plaque and the ability of these bacteria to colonise tooth surfaces (Jordan & Hammond, 1972; Loesche & Syed, 1978).

The human isolate of A. viscosus, strain T14V, was reported to produce extensive plaque, root surface caries and bone destruction characteristic of periodontal disease in germ-free

rats (Hammond et al, 1976). Fitzgerald et al (1981) used this same strain to induce periodontal disease in conventionally reared BALB/c mice. This mouse model of periodontal disease forms the basis of the current work and will be described in detail in a subsequent chapter. As it had been shown that Actinomyces species are especially potent lymphocyte stimulators and are thus implicated as important agents in the periodontal disease process (Ivanyi & Lehner, 1971a), Fitzgerald et al (1981) also studied the immune response, both cellular and humoral, to the organism. Their findings will be compared and contrasted with those of the current work in subsequent chapters.

1.2.6 CONCLUDING REMARKS

The fact that animal models in general, and mice in particular, have a valuable role to play in periodontal disease research is recognised by many investigators, but this is only true if the special physiological changes found in these animals are fully appreciated. Most certainly, the many advantages found in such an easily manipulated model should generate important information it would not otherwise be possible to obtain.

1.3 OBJECTIVES OF THE STUDY

The overall objective of the present study was to investigate the roles of different components of the host defence system in determining susceptibility to periodontal disease in the mouse. A number of approaches were envisaged:-

(a) Using mutants with specific disorders of the immune system. A number of these are available including mutants with specific defects of T-cells, B-cells, complement components and polymorphonuclear leucocytes and with diabetes.

(b) Studying naturally occurring disease in these mutants and, particularly, disease induced by oral inoculation with live A. viscosus.

(c) Studying the host response in terms of:

(i) Alveolar bone loss in gross defleshed specimens of the jaws to assess periodontal disease experience. This could be used to monitor disease progression with time in animals both exposed and not exposed to A. viscosus.

(ii) Histological sectioning and staining of fixed jaw specimens to investigate the tissue response. These studies could be used to describe the presence of plaque, calculus, hair or foreign body impaction, and the presence and degree of inflammatory infiltrate, loss of epithelial attachment, pocket formation and alveolar bone loss. Findings could be related to alveolar bone loss in the gross specimens.

(iii) Polymorphonuclear leucocyte function tests, consisting

of chemiluminescence studies to provide measures of general cellular function and myeloperoxidase assays as a more specific test of bactericidal potential. These tests would be performed on peritoneal exudate neutrophils.

(iv) Lymphocyte transformation to indicate the importance of the cellular component of the immune system. Splenic cells could be used as a measure of responsiveness of the systemic immune system and mesenteric lymph node cells as a measure of responsiveness of the gut-associated immune system. In addition, study of the stimulation and/or suppression induced by each system on the other would be possible using co-cultures.

(v) Serum immunoglobulin studies to determine the role of the humoral components of the immune system. The levels of non-specific immunoglobulins of the classes IgG, IgA and IgM, and of specific anti-A. viscosus immunoglobulin in any mice inoculated with this organism, could be studied.

(d) Microbiological studies to investigate the naturally occurring oral flora and the results of oral inoculation with live A. viscosus. As this organism is found only in relation to teeth, estimation of carriage/colonisation would be performed by removal of the molar teeth, grinding these in growth medium, and plating out the resultant suspension.

From the results of these various investigations, it was envisaged that it would be possible to reach some conclusions as to the relative importance of different components of the host defence system in controlling susceptibility to periodontal disease in the mouse. In this way, it was hoped

that new clues might be provided for solutions to the many unanswered questions which exist in relation to human periodontal disease.

C H A P T E R 2

MATERIALS, GENERAL METHODS AND PRELIMINARY EXPERIMENT

2.1 M I C E

A number of different genotypes were used in the experiments described in subsequent chapters. Three inbred strains and three mutants were involved. The inbred strains were BALB/c, C57BL/6/Ola (C57) and CBA/Ca/Ola (CBA), and the mutants beige (bg), obese (ob) and X-linked immune deficiency (xid). The choice of mice was made on the basis of ease of availability and to provide a range of genotypes and defects of immune function.

2.1.1 INBRED STRAINS

The three strains selected have different origins and consequently are genetically distinct. Each strain has been maintained by brother-sister mating for at least 15 generations so that members of the same strain can, for practical purposes, be regarded as genetically identical.

BALB/c: The BALB/c mice were obtained from a breeding colony at the Faculty Animal Area, University of Edinburgh. Progenitors of these mice arrived there from Lac Ltd in 1972 (Olac Manual, OLAC Ltd, Oxon, UK).

C57: These mice were purchased from OLAC Ltd. The original C57 mice were derived by Little in 1921 from a mating between female 57 and male 52 from Miss Lathrop's stock. Sublines 6 and 10 were separated prior to 1937 and animals from Subline 6 were sent to the Jackson Laboratory (Bar Harbor, Maine, USA), and thence to the Agricultural Research Council (Compton, UK). OLAC first obtained these animals in 1976 (Olac Manual).

CBA: Mice of the strain CBA were also purchased from OLAC Ltd. The first CBA mice were derived in 1920 by Strong from a mating

between a Bagg Albino female and a DBA male. The Ca subline was sent to the Jackson Laboratory and thence to Haldane and to Grüneberg in 1932. OLAC first obtained animals of this strain in 1976 (Olac Manual).

2.1.2 MUTANTS

2.1.2.1 Beige

The strain used was obtained from OLAC Ltd, Oxon, UK, and is designated C57BL/6-bg/bg/Ola. The mutation arose spontaneously at Oak Ridge Laboratory (Tennessee, USA) and was back-crossed to C57BL/6 in 1961 at the Jackson Laboratory (Bar Harbor, Maine, USA). In 1980, the strain was sent to the OLAC Breeding Unit and, in 1981, to OLAC isolators after caesarian section, where the line has been maintained by brother-sister matings (Olac Manual, OLAC Ltd).

Beige (bg) is an autosomal recessive mutation causing a condition analogous to the Chediak-Higashi syndrome in man. It is so-called because one of its effects is on coat colour. Humans and experimental animals with this disorder are known to have a number of defects. Included is defective granule formation in a range of cell types (Padgett et al, 1970). In a survey carried out by Oliver and Essner (1973), it was found that 15 out of 23 tissues of the beige mouse had giant lysosomes detectable by histochemical localisation of acid phosphatase activity. Of these, the greatest aberrations occurred in liver, brain, kidney and granulocytes. In some tissues, such as kidney proximal tubule

cells, the giant lysosomes reached a size of up to 11 microns in diameter (Brandt et al, 1975) taking up most of the total cell volume. The mechanism of formation of the giant lysosomes is uncertain, although electron microscopic studies of developing leucocytes suggest that an abnormally high rate of uncontrolled fusion of normal lysosomes in younger cells occurs to form the giant lysosomes in older cells. The basis for this is unknown, although it could involve reported altered membrane composition or microtubule function, both of which could affect lysosomal biogenesis (Davis et al, 1971; Oliver & Essner, 1975).

An interesting alternative mechanism is suggested by the work of Essner and Oliver (1974) who found that the specialised region of smooth endoplasmic reticulum, which may be the site of biosynthesis of lysosomes, was abnormally dilated in the hepatocytes of beige mice. Abnormally enlarged nascent lysosomes were detected in this region, indicating that the morphological aberration may be the result of a defect at the site of biosynthesis of primary lysosomes. In addition, the fact that cholinergic agents correct the lysosome defect in rapidly dividing beige cells such as fibroblasts, but do not in more slowly turning over cells such as macrophages, is consistent with altered morphology arising from defective biosynthesis (Oliver et al, 1976). If lysosomal biosynthesis is altered, however, it is probably a qualitative rather than a quantitative defect, since studies have shown normal rates of synthesis of at least one lysosomal enzyme, beta-glucuronidase, in beige mice (Brandt & Swank, 1976; Swank et al, 1978).

Other defects found in the Chediak-Higashi syndrome in man are decreased bactericidal capacity of granulocytes (Root et al, 1972), decreased fusion of lysosomes with phagosomes (Root et al, 1972), decreased chemotaxis (Gallin et al, 1975) and platelet storage disease (Buchanan & Handin, 1977; Boxer et al, 1977). Affected individuals suffer repeated bouts of infection and often enter an advanced lymphoproliferative phase. As a result, few survive their teens. In human Chediak-Higashi patients and in beige mice, there is also a defect in cytolysis mediated by natural killer (NK) cells (Roder 1979; Haliotis et al, 1980), which normally spontaneously lyse tumour or virus-infected cells in vitro without prior immunisation (Herberman & Holden, 1978; Santoli & Koprowski, 1979). Roder and co-workers (Roder, 1979; Roder & Duwe, 1979; Roder et al, 1979) showed that impaired cytolysis was neither due to altered organ distribution or frequency of target binding NK cells nor to a change in target selectivity, and concluded that the NK defect seen in beige mice involved the final lethal hit of the NK cell against its target.

A number of authors (Stutman et al, 1978; Paige et al, 1978; Kumar et al, 1979; Burton, 1980; Burton et al, 1980, 1981; Lust et al, 1981; Stutman & Cuttito, 1981) have shown that there is heterogeneity among murine NK cells. One class of NK cell, NK_A, displays preferential cytotoxicity towards lymphoid tumour targets, is sensitive to treatment with specific anti-NK antiserum and complement, and shows absent or reduced activity in ^{89}Sr-treated mice (Kumar et al, 1979; Burton, 1980; Burton et al, 1980; 1981). A second class of NK cell preferentially lyses adherent

solid tumour targets and is resistant to treatment with specific anti-NK alloantisera and complement. Normal activity of these latter cells has been reported in ^{89}Sr-treated mice and these cells have been termed natural cytotoxic (NC) or NK_B cells (Kumar et al, 1979; Burton et al, 1981; Lust et al, 1981).

It has been shown that NK_B cell activity is normal in beige homozygotes (Burton et al, 1981; Lust et al, 1981; Stutman & Cuttito, 1981). On the other hand, impaired NK_A activity of spleen cells against lymphoma targets has been found in beige homozygotes although this abnormality could be partially or wholly overcome by prolonging the assay time (Bartlett & Burton, 1982).

The relatively unchecked growth of implanted tumours in beige mice has suggested that NK cells carry out tumour surveillance in normal individuals (Talmadge et al, 1980; Karre et al, 1980). As for protection against viral infection, little was known about the role of NK cells till the work of Shellam et al (1981) on the NK reaction to murine cytomegalovirus (MCMV) was published. While adoptive protection against MCMV can be conferred by T cells from immune mice (Starr & Allison, 1977; Ho, 1980), the primary cytotoxic T cell response is difficult to detect (Ho, 1980) and significant cytotoxicity is not observed before the fourth day of infection (Quinnan et al, 1980). Thus, while susceptible mice die within 3-4 days of infection and mice of resistant strains survive (Chalmer et al, 1977), protection of resistant mice by an early non-T cell mechanism appears likely.

In support of an early protective role for NK cells, it has

been shown that their cytotoxicity is rapidly enhanced during MCMV infection and that they lyse MCMV-infected cells in vitro (Quinnan & Manischewitz, 1979; Bancroft et al, 1981). A significant positive correlation between resistance to lethal MCMV infection and the level of NK cytotoxicity has been demonstrated at one day after infection in a number of mouse strains (Bancroft et al, 1981). Shellam et al (1981) reported that NK deficient beige mice were more susceptible to MCMV than heterozygous litter mates. This increased susceptibility to viral infection in beige mice strongly suggests that NK cells have a protective role in early MCMV infection.

In 1976, Lavine et al demonstrated that mutant mink suffering from the Chediak-Higashi syndrome showed a far greater severity of periodontal bone loss than their non-mutant counterparts. At the end of this publication, it was suggested that beige mice may be similarly afflicted. This influenced the choice of beige mice for the present study as it was anticipated that they would show a high level of spontaneous disease.

2.1.2.2 Obese

The strain obtained from OLAC Ltd, Oxon, UK, is designated C57BL/6-ob/Ola. The mutation arose spontaneously in an outbred multiple recessive stock prior to 1950 at the Jackson Laboratory (Bar Harbour, Maine, USA). In 1978, these animals were sent to the OLAC breeding unit after caesarean derivation (Olac Manual, OLAC Ltd, Oxon, UK).

Obese (ob) is an autosomal recessive mutation which, on the C57BL/6 background, causes marked obesity, hyperphagia, transient

hyperglycaemia and markedly elevated plasma insulin concentration (10-50 times normal) associated with an increase in the number and size of the beta-cells of the islets of Langerhans (Coleman, 1982). The obesity is of the hypertrophic-hyperplastic type, both the size and number of adipocytes being increased (Johnson & Hirsch, 1972). This is in contrast to most other rodent models of obesity where adipocyte enlargement alone is responsible for the massive fat stores (Coleman, 1982). Homozygotes of both sexes are infertile and must be obtained either by mating heterozygotes or by mating male heterozygotes to normal females into which ovaries from mutant obese females have been transplanted (Coleman, 1982). Biochemical studies have shown that the activities of most insulin-dependent enzymes are increased, the rate of liver and adipose tissue lipogenesis is more than twice normal, and that marked insulin resistance is associated with a down-regulation in the numbers of insulin receptors in several tissues (Chang et al, 1975; Soll et al, 1975). This loss of receptor sites is associated with progressive hyperglycaemia and seems general in all obesity mutants studied (Coleman, 1982). Down-regulation of receptor site may be a mechanism whereby the mutants are protected against lethal hypoglycaemia which would occur if the high concentrations of circulating insulin were fully effective. Those enzymes involved in gluconeogenesis that normally are decreased in the hyperinsulinaemic state remain elevated in obese mice and probably contribute to the hyperglycaemia. Increased gluconeogenesis occurs early in development in several obesity mutants and may be associated with the loss of insulin receptor sites in specific target cells (Coleman, 1982).

The obese mouse has many defects that suggest a hypothalamic disorder. These include hyperphagia, hyperinsulinaemia, infertility and defects in thermoregulation (Coleman, 1982). Despite the hyperphagia, obese mice are remarkably efficient with regard to utilization of food. Mutants can maintain weight and deposit excess lipid even when food intake is restricted to 50% of the normal mouse requirement. The factors causing this increased metabolic efficiency are unknown but, when established, could have major implications regarding how different modes of energy utilisation contribute to the development of diabetes and obesity.

Of the factors implicated in periodontal disease, abnormalities of collagen and cell-mediated immunity have been studied in obese mice. A deficiency of collagen formation during wound healing has been reported (Goodson & Hunt, 1979), and skin collagen has shown abnormal mechanical and chemical properties (Enser & Avery, 1984). Thompson et al (1983) found resistance to melanoma metastasis and an enhanced T-lymphocyte mitogenic response, while Meade et al (1979) reported that the ability of spleen cells to kill mastocytoma target cells was impaired in obese mice. Similarly, Chandra (1980) reported that NK activity was increased while Clark et al (1981) found that it was reduced in these mutants. Further investigation of the immune responses of obese mice is therefore indicated. There is some suggestion that any abnormality of cell-mediated immunity may be the result of the metabolically abnormal tissue environment rather than intrinsic to the immune system itself (Meade et al, 1979; Chandra, 1980).

2.1.2.3 X-linked immune deficiency

The strain obtained from OLAC Ltd, Oxon, UK, is designated CBA/HN/Ola. The origin of strain CBA was as described above. The H subline reached the National Institutes of Health, Bethesda, USA, in 1966, where the X-linked immune deficiency (xid) mutation was discovered (Olac Manual, OLAC Ltd).

Hemizygous males and homozygous mutant females are affected. The defect is characterised by inability to mount an antibody response to various helper T cell-independent (TI) antigens, low immunoglobulin (Ig)M antibody responses to some helper T cell-dependent antigens, as well as low serum IgM and IgG_3 levels (Amsbaugh et al, 1972; Scher et al, 1973; Amsbaugh et al, 1974; Press, 1981; Scher, 1982a). B cells of xid mice express a low ratio of IgM to IgD surface Ig (Finkelman et al, 1975) and are extremely susceptible to the induction of immunological tolerance (Metcalf et al, 1980). They also lack Lyb-3,5 and 7 alloantigens which are normally expressed by mature B cells (Huber et al, 1977; Ahmed et al, 1977; Subbarao et al, 1979). Mosier et al (1977) proposed that TI antigens could be divided into two categories, TI-1 antigens which elicit an immune response in xid mice and TI-2 antigens which do not.

B lymphocytes from normal mice comprise a mixture of mature and immature cells (Scher, 1982b; Mond, 1982; Huber, 1982). Immature B cells arise early in ontogeny, express a high density of surface IgM, lack Lyb-3,5 alloantigens, respond to TI-1 and not TI-2 antigens and reside largely in the spleen. Mature B cells develop late in ontogeny, express Lyb-3,5 alloantigens and a low

density of IgM, respond to TI-2 and not TI-1 antigens and occupy both spleen and lymph nodes (Sprent et al, 1985). Whether these two subsets represent distinct lineages or different developmental stages of the same lineage remains unclear. In attempting to resolve this question, much emphasis has been placed on studying the properties of xid B cells, which exhibit many of the properties of immature B cells of normal adult mice. This has given rise to the notion that xid mice are selectively devoid of mature cells, the lack of these cells reflecting either a stem cell defect in the mature B cell lineage or a block in the differentiation of immature to mature B cells (Huber, 1982).

However, Sprent et al (1985) showed that, in terms of cell distribution in the lymphoid tissues, homing properties, capacity to recirculate and rate of turnover, the majority of xid B cells are indistinguishable from the mature B cells of normal mice. On the other hand, in terms of total numbers, these authors found that xid mice contained only about one-third of the number of B cells recovered from non-defective mice. Significantly though, this quantitative deficiency of B cells in xid mice was no more marked in lymph node or thoracic duct lymph than in spleen, implying that the ratio of recirculating cells to sessile (or spleen-seeking) cells in xid mice is no less than in normal mice. Evidence for long-lived circulating B cells in xid mice has come from recent studies by Chaudhuri et al (1983).

Work by Kiyono et al (1983) investigated the secretory immune system in xid mice. Their experiments showed that the gut-associated lymphoreticular tissue (GALT), such as Peyer's patches

(PP), possessed unique populations of T cells which supported *in vitro* responses in xid B cell cultures from both the spleen or PP, and thus directed mature B cell populations present towards IgA isotype specific responses. While the magnitude of the response in xid mice was found to be lower than in similarly treated non-xid controls, the work showed the xid secretory immune system to be operative. This is clearly important in any experimentation involving oral antigen administration.

2 1.3 MAINTENANCE AND CROSSES

All the mice were maintained at a constant temperature of 70°F and housed at a maximum of 10 animals per cage. Food and water were given *ad libitum* except as stated otherwise in the experimental schedule. A 12 hours light and 12 hours darkness cycle was automatically controlled. Uninoculated mice and mice inoculated with *A. viscosus* were kept in different rooms and the infected animals' food bowls and water bottles were washed separately to avoid cross-infection. All mice were weaned at three weeks of age. Both a standard laboratory diet and a special high carbohydrate/low fat diet were used (see Appendix).

Mice of the required genotypes were obtained as follows:

(a) BALB/c mice of the appropriate age were bought from a large breeding colony at the Faculty of Medicine Animal Area (University of Edinburgh).

(b) Both the C57 and bg/bg stocks were maintained first by breeding pairs of the originally purchased animals and subsequently by breeding pairs of their progeny.

(c) +/bg heterozygotes were produced by mating C57 males with bg/bg females.

(d) ob/ob mice were bought in the numbers required as the maintenance of obese stocks posed special difficulties.

(e) To obtain the different xid genotypes, 10 xid/xid females, 10 xid/Y males and 10 female and 10 male CBA mice were bought. Matings were as follows:-

(i)	Female	Male
Parents	xid/xid	+/Y(CBA)
Offspring	+/xid (discarded)	xid/Y(kept)

(ii)	Female	Male
Parents	+/+ (CBA)	xid/Y
Offspring	+/xid (kept)	+/Y (kept)

2.1.4 SUSCEPTIBILITY TO BACTERIAL PATHOGENS

A summary of previous work involving bacterial challenge of certain of the mouse genotypes used in the present study or related strains is shown in Table 2.1, though none of the organisms investigated previously have been considered periodontal pathogens. In each case (except Hector et al, 1982), resistance or susceptibility was judged according to whether or not the animals died as a consequence of inoculation with the named organism.

The clear conclusion is that there is variation in response of different genotypes to the same organism and of the same genotype to different organisms. There is some suggestion that CBA has tended to be less susceptible than the other two inbred strains, although this did not apply to infection by Candida albicans.

Studies of the progeny of crosses between strains susceptible and resistant to certain organisms have suggested the existence of autosomal dominant resistance genes (Wardlaw, 1970; Plant & Glynn, 1976; Gros et al, 1981; Brown et al, 1982). However, O'Brien et al (1979,1981) showed that susceptibility to Salmonella typhimurium infection can be X-linked.

Table 2.1 Summary of previous work involving bacterial challenge of mice of selected genotypes.

ORGANISM	MOUSE STRAIN	SUSCEPTIBILITY	INOCULATION ROUTE	REFERENCE
Candida albicans	BALB/cByJ	-	Subcutaneous and intravenous	Hector et al, 1982
	C57BL/6J	-		
	CBA/J	+		
Streptococcus pneumoniae	xid (CBA/N)	+	Intravenous	Yother et al, 1982 Briles et al, 1981
Corynebacterium kutscheri	C57BL/6	-	Intravenous	Hirst & Wallace, 1976
Mycobacterium lepraemurium	BALB/c	+	Intravenous	Brown et al, 1982
	C57BL	+		
	CBA/Ca	-		
Mycobacterium bovis	BALB/c	+	Intravenous	Gros et al, 1981
	C57BL/6J	-		
	CBA/J	-		
Salmonella typhimurium	BALB/c	+	Intraperitoneal or intravenous	O'Brien et al, 1979
	C57BL/6	+		
	C57BL/10	+	Subcutaneous	Plant & Glynn, 1976
	CBA/CaHN	-	Intraperitoneal or intravenous	O'Brien et al, 1981
	xid (CBA/N)	+		
Bordetella pertussis HSF (histamine sensitising factor)	BALB/cJ	+	Intraperitoneal	Wardlaw, 1970
	C57BL/6J	+		
	CBA/J	-		

2.2 MICROORGANISMS

2.2.1 INTRODUCTION

There are a number of pathogenic microorganisms that resemble both bacteria and fungi. Some of these are included in the order Actinomycetales and one genus in this order is Actinomyces, whose members are non-sporulating, filamentous, anaerobic or microaerophilic Gram-positive bacilli. These organisms are found as commensals in the oral cavity, the predominant species being Actinomyces israelii, the principal cause of human actinomycosis, Actinomyces naeslundii and Actinomyces viscosus (Burnett & Schuster, 1978). A. viscosus, in particular, has been implicated in both human and animal gingivitis and periodontitis.

2.2.1.1 Colonisation

A study by Socransky et al (1977) showed that Streptococcus sanguis and A. viscosus were predominant among the pioneer colonisers of newly cleaned tooth surfaces. Dextrans from several sources aggregated cells of some A. viscosus strains (Bourgeau & McBride, 1976) and some A. viscosus strains attached to S. sanguis by mechanisms that apparently did not involve dextrans (McIntire et al, 1978; Cisar et al, 1979). In vitro co-aggregation of A. viscosus and Veillonella strains in the form of corn-cob and bottle-brush aggregates is mediated by extracellular polymers synthesised by A. viscosus (Newbrun, 1979).

Further information on A. viscosus colonisation and aggregation comes from experiments on gnotobiotic rats. Rats monoinfected with strain T14-Vi suffered periodontal damage as assessed by bone loss while those infected with T14-AV did not

(Brecher et al, 1978). Electron microscopic studies of involved teeth showed much fewer T14-AV to be present than T14-Vi and further examination of the cell surfaces of each strain showed that T14-Vi possessed more surface fibrils than strain T14-AV. In addition, the fibrils present on strain T14-Av were embedded in an extracellular capsule (Powell et al, 1978). Perhaps the reduced number of surface appendages and their occlusion in extracellular material reduced the adsorption to, and subsequent colonisation of, tooth surfaces by T14-AV and resulted in lower pathogenicity relative to strain T14-Vi.

2.2.1.2 Gingivitis

Recent in vitro research into the surface adsorption of A. viscosus seems to point to the involvement of at least two antigenically distinct and functionally independent surface fimbriae. Monospecific antifimbrial IgG and Fab fragments and fimbrial deficient mutants of A. viscosus strain T14V have been used to show that type 1 fimbriae mediate the adsorption of A. viscosus strain T14V to saliva-treated hydroxyapatite surfaces in vitro (Clark et al, 1984; Clark, 1985). The type 2 fimbriae have been shown to mediate interbacterial aggregation between A. viscosus strains and other plaque bacteria such as Streptococcus sanguis (Cisar et al, 1979, 1981, 1983; Kolenbrander, 1982; Revis et al, 1982).

While functional activities have been associated with each type of A. viscosus fimbriae, these adhesions have not been structurally characterised because isolated fimbriae are resistant to complete dissociation (Cisar, 1986). Therefore, cloning of A.

viscosus genes in Escherichia coli was initiated as an approach to the identification of the fimbrial subunits. A type 2 fimbrial gene was cloned by Donkersloot et al in 1985 and a type 1 fimbrial unit was cloned by Yeung et al in 1987. It is hoped that these advances will facilitate further studies to increase our understanding of A. viscosus colonisation.

In the gingival crevice and periodontal pocket, there is a zone of plaque that is directly attached to the tooth or calculus surface. The organisms in this zone are mainly Gram-positive rods. Included is A. viscosus, reflecting its role in early colonisation. The development of gingivitis as such is, however, thought to be caused by the bacteria associated with the subsequent increase in supragingival and gingival margin plaque formation. The Gram-positive filamentous rods, mainly Actinomyces species, including A. viscosus, appear to be of major significance in this clinical condition (Slots, 1977).

In experimental gingivitis, there is an increase in the total mass of supragingival plaque as well as increased proportions of members of the genus Actinomyces. This genus tends to be dominant in supragingival plaque and frequently comprises 50% or more of the total bacterial isolates.

The bacteriology of human experimental gingivitis has been studied relative to plaque age (Syed & Loesche, 1978) and to plaque and gingivitis scores (Loesche & Syed, 1978). These authors showed that while streptococci dominated in early plaque, Actinomyces species, including A. viscosus, dominated in older plaques and were associated with gingival bleeding. These studies are important as

they suggest that proportional changes in the subgingival microflora may uniquely contribute to the development of gingival inflammation.

2.2.1.3 Periodontitis

In chronic periodontitis, there is a large amount of attached subgingival plaque (Listgarten, 1976) in which filamentous organisms such as A. viscosus are numerous (Socransky, 1977); Darwish et al, 1978). Experimental studies in gnotobiotic rats and pathogen-free hamsters suggest a degree of specificity of bacteriological involvement in the pathogenesis of this condition. The pathogenic potential of certain Gram-positive bacteria which formed plaque in gnotobiotic rats fed high sucrose diets was tested, and certain microorganisms, including A. viscosus, resulted in accelerated alveolar bone loss (Jordan et al, 1972; Guggenheim & Schroeder, 1974; Irving et al, 1974; Crawford et al, 1978a & b). In 1981, Fitzgerald et al published their method of inducing periodontal disease in conventionally maintained mice fed a high sucrose diet by oral inoculation with live A. viscosus.

When the skin of humans with periodontitis was tested with antigen prepared from A. viscosus, a positive hypersensitivity reaction was produced (Nisengard et al, 1968). Lymphocytes from patients with gingivitis and periodontitis exhibited a blastogenic response to antigens prepared from A. viscosus while lymphocytes from disease-free controls did not respond (Patters et al, 1976), thus implicating this organism in the pathogenesis of periodontal disease.

However, A. viscosus may not act in isolation. Recently it has

been reported that it is necessary for plaque to contain Actinomyces species and other Gram-positive bacteria for Bacteroides melaninogenicus, an important periodontopathogen, to become part of the resident oral microflora (Sidaway, 1979).

2.2.2 AIMS

A. viscosus was to be used to induce periodontal disease, along the lines described by Fitzgerald et al (1981), in host mice of different genotypes. Accordingly, there were three main aims of the bacteriological work:

(a) Successful culture and identification of the organism by morphological and biochemical means.

(b) Preparation of an antigen from the organism to be used in subsequent immunological tests.

(c) Preliminary investigation of the antigen produced.

2.2.3 TECHNIQUES

2.2.3.1 Source and stock

The organism used was Strain 10951 of Actinomyces viscosus obtained as lyophilised cultures from the National Collection of Type Cultures, London (NCTC). Lyophilised cultures were reconstituted with a few drops of Cooked Meat Broth (CMB: see Appendix), then transferred to 10 ml of the same medium. After aerobic incubation for 24 hours at 37°C these CMB cultures remained at room temperature and served as stock cultures for further tests.

2.2.3.2 Culture

As A. viscosus is described as microaerophilic, initial

culturing was performed under both aerobic and anaerobic conditions, with the latter carried out using anaerobic jars and the Gas Pak system (Becton Dickinson UK Ltd), a disposable carbon dioxide and hydrogen generator. Visual assessment of growth by turbidity checks comparing the two modes of culture showed that aerobic culture produced greater growth. Aerobic culture was therefore used for all subsequent work. Both liquid- and solid-phase culture was performed.

Liquid-phase culture was carried out by adding three drops of CMB stock culture to proteose peptone yeast medium (PPY: see Appendix) supplemented with 1 ml 2% sodium carbonate and 1 ml 3.75% cysteine hydrochloride per 50 ml PPY. This was then incubated for 48 hours at 37°C. Each time such a culture was produced, a purity check was carried out by plating one drop of the culture on blood agar and examining all colonies in terms of gross morphology and Gram staining.

Solid-phase Culture was carried out on two types of agar plate:

(a) Blood agar, prepared by adding out-dated human blood to Columbia agar base (Oxoid Ltd, London, UK) to a concentration 5% ($^{v}/v$).

(b) Semiselective agar, produced as above with the addition of 10 mg colistin sulphate and 15 mg nalidixic acid per litre agar base.

In both cases, 15 ml of agar was dispensed into each plate and plates were either used fresh or stored for up to one day at 4°C. After plating out on either medium, incubation at 37°C for 48 hours resulted in recognisable growth.

2.2.3.3 Identification

A. viscosus NCTC 10951 was identified by a number of tests used to monitor its presence throughout the various experiments described in subsequent chapters:-

(a) Morphology on agar plates

Colonies on blood agar were flat, smooth, greyish in colour, and approximately 2 mm in diameter. On the semiselective agar, they were more domed in shape and were whiter in colour, but again of size approximately 2 mm in diameter. On both media, alpha-haemolysis was seen.

(b) Gram stain

Gram stain produced an appearance of Gram-positive bacilli.

(c) Biochemical tests

To perform these, a few drops of CMB stock culture were placed in 20 ml supplemented PPY medium and incubated at 37°C overnight to produce a working culture. From this, 1 ml aliquots were taken to perform the following tests:

(i) Sugar Fermentation Tests:- Here the culture was added to sugar containing tubes to produce a 1% final concentration of ribose, raffinose, mannitol or glucose. A control tube containing no sugar was also included and all tubes were incubated aerobically for 24 hours at 37°C. After this time, tube pH changes were recorded either by pH meter or by addition of 30 µl 0.01% bromothymol blue indicator. A pH decrease in excess of 0.5 pH unit or a change to yellow colour relative to control was considered a positive result.

(ii) Urease test:- The culture was added to a tube containing

urea to a final concentration of 1%. After 24 hours of incubation at 37°C, the pH change was recorded either colorimetrically or by pH meter. Production of a blue colour or an increase in pH of 0.5 pH unit relative to control was considered a positive result.

(iii) Catalase test:- A few drops of 10% $^{v}/v$ hydrogen peroxide were added to 1 ml of the culture. Bubble production indicated a positive response.

(iv) Nitrate test:- 200 $\mu g\ ml^{-1}$ potassium nitrate was added to 1 ml of the culture and incubated aerobically at 37°C overnight. The next day 0.2 ml of Nitrate Test Solution 1 was added, followed by 0.2 ml of Nitrate Test Solution 2 (see Appendix). The development of a red colour was taken to indicate a positive result.

(v) Gas-liquid chromatography (GLC):- Cherry and Moss (1969) described gas-liquid chromatography as a rapid and sensitive technique for the analysis of certain microbial cell components or their products that would aid taxonomists and clinicians. Certain short chain volatile fatty acids are characteristic of many organisms (eg. propionic, butyric, isobutyric, valeric and isovaleric acids), although GLC profiles cannot alone identify an organism to species level (Holdeman et al, 1977). A number of authors have reported the effects of growth medium composition on GLC profiles of metabolic fatty acids (Deacon et al, 1978; Lombard, 1979; Turton et al, 1983a & b). However, as all the organisms used in this study were cultured in supplemented PPY, this problem was avoided. A 48 hour PPY culture of A. viscosus was produced as described earlier, and five drops of 50% sulphuric

acid were added to 5 µl of culture and well mixed. The culture was then centrifuged at 1000 g for 15 minutes and the supernatant collected for analysis of volatile fatty acids. Methyl derivatives of non-volatile fatty acids were prepared according to Holdeman et al (1977). Samples (1 µl) were then analysed by GLC using a column of SPIZZO on chromosorb WAW (Supelco Inc, Belefonte, Pennsylvania, USA) at a temperature of 147°C.

Gas flow rates were as follows:-

oxygen free nitrogen (the carrier gas)	35 ml min^{-1}
hydrogen	35 ml min^{-1}
air	525 ml min^{-1}

A Pye Unicorn 104 chromatograph with a flame-ionisation detector (200°C) was used. Standard solutions containing volatile and non-volatile fatty acids were injected into the column for comparison prior to samples. The concentration of a particular acid in a sample was calculated, from the size of the corresponding peak traced on a recorder, by the following equation:-

Concn. of acid = Peak height of test/Peak height of standard

The results of biochemical tests on A. viscosus NCTC 10951 are shown in Table 2.2.

Table 2.2 Results of biochemical tests on A. viscosus NCTC 10951

Sugar Fermentation:

Sugar	pH Change 0.5 pH Unit	Colour Change
Ribose	-ve	-ve
Raffinose	+ve	to yellow
Mannitol	-ve	-ve
Glucose	+ve	to yellow

Urease Test: pH increase 0.5 pH unit relative to control and a colour change to blue

Catalase Test: Positive

Nitrate Test: Positive

GLC: The fatty acid end products of metabolism for A. viscosus were:

Acetic acid	4.7)	
Lactic acid	18.8)	moles ml^{-1}
Succinic acid	4.4)	

2.2.3.4 Comparison of Actinomyces viscosus recovery from blood agar (BA) and semiselective agar (SSA) plates

Successful culture and identification of A. viscosus was essential if disease induction via oral inoculation of the organism was to be possible. If pure cultures of A. viscosus were to be used exclusively, ordinary blood agar plates for solid phase culture would have been adequate. However, it was envisaged that recovery of A. viscosus from mixed flora would be required in subsequent experiments, so use of a semiselective agar (SSA) to facilitate this was deemed appropriate. The addition of colistin sulphate and nalidixic acid does not permit the growth of many Gram-negative organisms. Any such selective agar is often, however, at least partially inhibitory to most other organisms. It was therefore important to determine whether the inhibition would be too great for adequate growth of A. viscosus NCTC 10951.

A 48 hour culture of A. viscosus was prepared as previously described and a bacterial count performed using a 0.1 mm depth Thoma Ruling Cell Count Chamber (Hawksley Ltd, Lancing, UK). This suspension underwent a 10^{-5} dilution, and 100 ul of this was plated out on each of five blood agar and five SSA plates. The plates were then aerobically incubated at 37°C for 48 hours and the resultant colonies counted.

The original cell count of the 48 hour culture was 1.09 x 10^9 cells ml^{-1}. Thus, plating of 100 ul at a 10^{-5} dilution resulted in plate reception of 1090 potential colony forming units (cfu) per plate. The counts produced on each of the five plates of each medium were:

	BA	SSA
	902	532
	704	540
	953	587
	834	505
	832	503
Mean + s.e.	845 ± 42	533 ± 15

Mean as a proportion of plate reception:

for BA = 78% for SSA = 49%

There was thus a statistically significant difference in colony forming ability between the two media. However, inhibition by SSA was not too great to allow adequate growth of A. viscosus and so, in all subsequent recovery experiments, the semiselective agar was used. The use of blood agar was restricted to purity checks of cultures.

2.2.3.5 Preparation of an EDTA antigen extract from *A. viscosus*

This antigen was produced using a modification of the method described by Poxton and Brown (1979). As often as required, two litres of 48 hour *A. viscosus* culture in supplemented PPY medium was prepared as previously described and, after purity checks, the cells were washed three times in phosphate buffered saline (PBS) with the first wash involving spinning the cells at 17,500 g for 10 minutes and the following two at 20,000 g for 10 minutes. The pellets produced after the third wash were resuspended in 2 ml each of 10 mM ethylene-diamine-tetraacetic acid (EDTA) at neutral pH and placed in glass tubes in a 45°C water bath for one hour. This suspension was then centrifuged at 10,000 g for three minutes and the supernatant containing the antigen extract transferred to acid-washed Bijou bottles. The protein content of individual EDTA extracts was estimated by the Lowry method (Lowry et al, 1951) using 2 mg ml^{-1} bovine serum albumin as the standard.

2.2.3.6 SDS PAGE of EDTA *A. viscosus* extract

In recent years, a technique that has received much attention as an aid to taxonomic studies of bacteria is polyacrylamide gel electrophoresis (PAGE). This procedure involves the separation of cell extracts, usually proteins, according to molecular size by the sieve-like effects of the porous acrylamide gel, and their visualisation by staining with, for example, Coomassie blue, silver or amido black. Such proteins are made soluble with sodium dodecyl sulphate (SDS) which is the anionic detergent used to resuspend samples before their application to gels and is also

included in electrophoresis buffers and gels themselves. SDS dissociates proteins into monomeric polypeptides and coats them with a uniform negative charge, thus promoting their migration towards the anode during electrophoresis. The rate of migration of these molecules, and therefore their final position in the stained gel, is determined by their molecular weight and the gel pore size. An additional function of SDS is to prevent re-aggregation of polypeptides into their native configurations. Polyacrylamide gels consist of acrylamide and methylene bis-acrylamide, polymerised by the addition of ammonium persulphate and tetra-methyl-ethylene-diamine. Two types of gel are used, gradient gels, in which the concentration of acrylamide increases away from the point of sample application or, more commonly, and as was used in this study, homogeneous gels.

A modified version of the method of Laemmli (1970) was used as described by Poxton and Brown (1979). Vertical slab gels (170 x 140 x 1 mm) consisted of a 10 mm 4% acrylamide stacking gel above a 10% acrylamide separating gel. Samples of the EDTA extract containing 100 ug protein per 100 ul single strength sample buffer (containing SDS and boiled for three minutes) were added to a well in the stacking gel and run under the following conditions. 60 V was applied to the gel to move samples through the stacking gel (1-2 hours) followed by 150 V until the sample buffer front (indicated by bromophenol blue) was approximately 2 cm from the bottom of the separating gel (2-3 hours). Gels were then stained with Coomassie blue (Poxton & Sutherland, 1976).

A photograph of an SDS PAGE separation of the EDTA

A. viscosus extract is shown in Figure 2.1. Many bands are present, confirming that the antigen was a crude mixture of fragments.

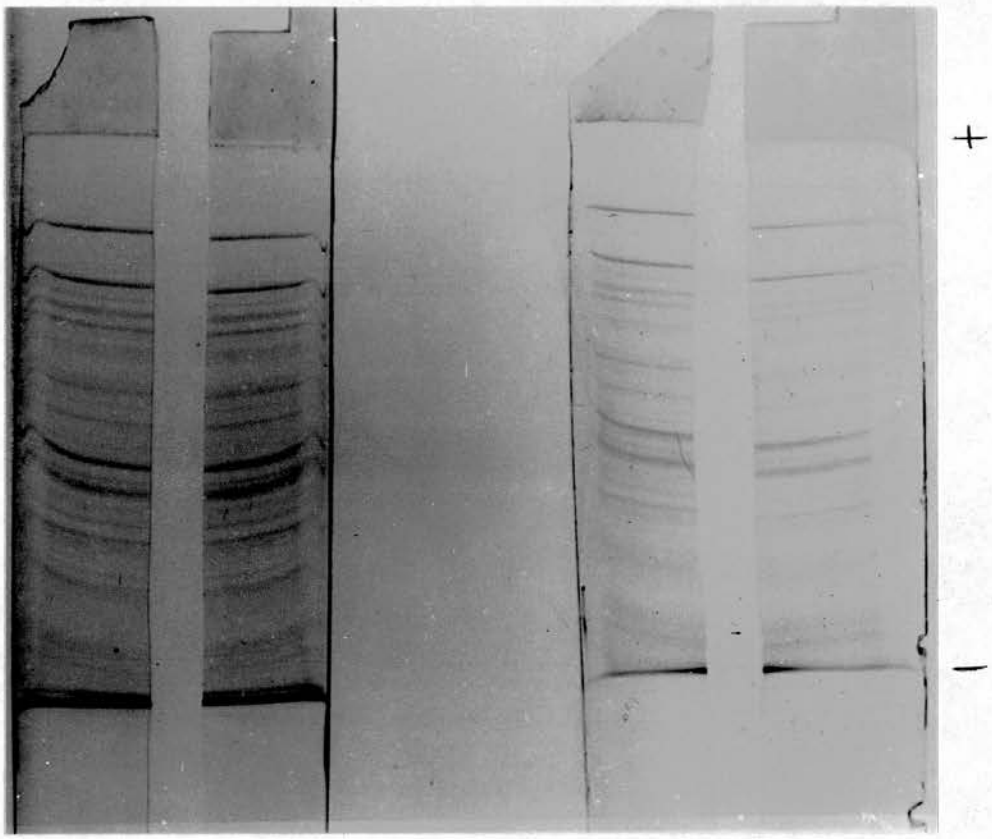


Fig 2.1 SDS PAGE separation of the EDTA A. viscosus extract stained with Coomassie blue.

2.3 PRELIMINARY EXPERIMENT

2.3.1 INTRODUCTION

This experiment confirmed a similar piece of work reported by Fitzgerald et al in 1981. These authors found that oral inoculation of A. viscosus resulted in colonisation of the teeth only, and that recovery of the organism could be achieved by grinding the molars of infected animals in growth medium and plating out the resultant suspension. The experiment was carried out in the present study:

(a) to practise the oral inoculation procedure;

(b) to determine appropriate dilutions and volumes of molar suspensions to plate out for colony counting;

(c) to determine whether the age of mice at the time of inoculation affected subsequent organism recovery;

(d) to confirm that the method used resulted in recovery of an organism indistinguishable from that originally inoculated.

2.3.2 MATERIALS AND METHODS

The experiment was performed according to the schedule shown in Figure 2.2.

Five BALB/c mice were obtained at the following ages: 3 weeks, 11 weeks and 19 weeks. All the mice were fed, ad libitum, the special high carbohydrate low fat diet used in subsequent experiments (see Appendix).

A 48-hour culture of A. viscosus in supplemented PPY medium was prepared as described previously. The number of organisms ml^{-1} was then determined and the culture adjusted to 3.2×10^8

organisms ml^{-1}. Seven days after instituting the special diet, one 50 µl aliquot of the A. viscosus suspension, containing 1.6 x 10^7 cfu, was placed in the mouth of each of the 15 mice using a micropipette (Anachem Ltd, Luton, UK) with a fresh sterile disposable pipette tip (Flow Labs, Rickmansworth, UK) for each inoculation. The animals were left without water overnight and the inoculation procedure repeated with fresh 48-hour cultures on the two successive days. The mice were then given food and water ad libitum.

Seven days after completion of the inoculation procedure, one mouse per group was sacrificed by ether inhalation and its molars were removed. The three molars from each quadrant of each mouse were ground in 2 ml supplemented PPY medium using a tissue homogeniser. Serial dilutions of each suspension were made to produce four working dilutions; 0, 10^{-1}, 10^{-2}, 10^{-3}; and 200 µl of each of these was plated in triplicate on SSA. Plates were incubated aerobically at 37°C for five days and examined for A. viscosus growth. After this time, visual evaluation was used to determine the appropriate dilution for the remaining mice of each age group. The remaining four mice per group were sacrificed by ether inhalation 12 days after completion of the inoculation procedure. Molars were removed by quadrant and ground in 2 ml of supplemented PPY as before. 200 µl of the appropriate dilution suspension (as determined from the single mouse of each group) was plated in triplicate on SSA and incubated aerobically at 37°C for five days. Colony counts were then made and random samples of colonies subjected to the identification procedures described previously.

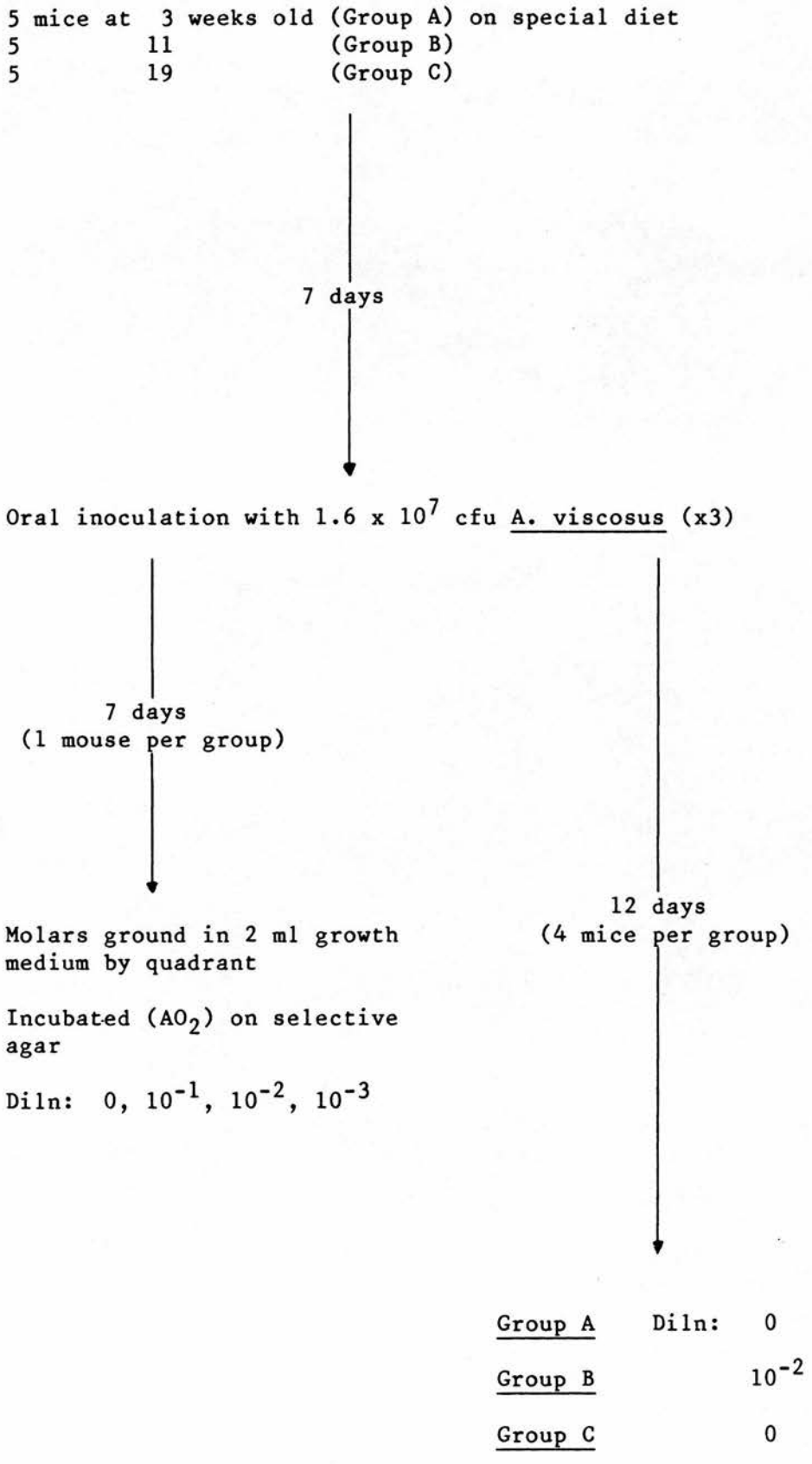


Figure 2.2 Diagrammatic representation of the schedule used for the preliminary experiment.

2.3.3 RESULTS

Serial dilutions from the single mouse of each group showed that the appropriate dilutions by age at inoculation were:

Age at inoculation (weeks)	Dilution
4	0
12	10^{-2}
20	0

Colony counts were as given in Table 2.3, and the differences in recovery with age at inoculation are summarised in Figure 2.3. The peak at 12 weeks was so extreme that a formal test of statistical significance was not required.

The identification procedures showed that the organism recovered was indistinguishable from that inoculated.

2.3.4 DISCUSSION

The A. viscosus recovery procedure necessarily resulted in a culture of mixed oral floras on the agar plates. An incubation time of five days proved to be required to produce adequate growth of A. viscosus colonies on these plates compared with two days for those receiving only a pure culture of A. viscosus. It must therefore be assumed that the other organisms have a partially inhibitory effect on A. viscosus growth. In all subsequent recovery experiments plates were therefore incubated for five days.

The identification procedures showed the organisms recovered to be indistinguishable from that originally inoculated. This confirms that the colonies cultured were truly A. viscosus and not another related actinomycete and shows that new strains of the organism did not develop in the mouse mouths with time.

Table 2.3 Means of triplicate colony counts per mouse quadrant for different ages at inoculation. UR, UL, LR, LL = upper right, upper left, lower right, lower left.

Age at inoculation (weeks)	Mouse	cfu per quadrant ($x10^3$) UR	UL	LR	LL	Mouse Mean
4	1	2.36	2.22	2.36	2.23	2.29
4	2	2.23	2.25	2.12	2.42	2.25
4	3	2.30	2.26	2.08	2.38	2.25
4	4	2.37	2.44	2.07	2.38	2.31
	Quadrant Mean	2.31	2.29	2.16	2.35	Overall Mean = 2.27×10^3

Age at inoculation (weeks)	Mouse	cfu per quadrant ($x10^5$) UR	UL	LR	LL	Mouse Mean
12	1	1.44	1.40	1.45	1.45	1.44
12	2	1.42	1.50	1.39	1.42	1.43
12	3	1.38	1.41	1.32	1.35	1.36
12	4	1.44	1.37	1.40	1.44	1.41
	Quadrant Mean	1.42	1.42	1.39	1.41	Overall Mean = 1.41×10^5

Age at inoculation (weeks)	Mouse	cfu per quadrant ($x10^3$) UR	UL	LR	LL	Mouse Mean
20	1	1.78	2.08	1.69	2.30	1.96
20	2	0.83	1.05	1.04	1.57	1.12
20	3	1.63	1.18	1.59	1.95	1.59
20	4	0.78	1.34	1.54	0.88	1.14
	Quadrant Mean	1.26	1.41	1.46	1.67	Overall Mean = 1.45×10^3

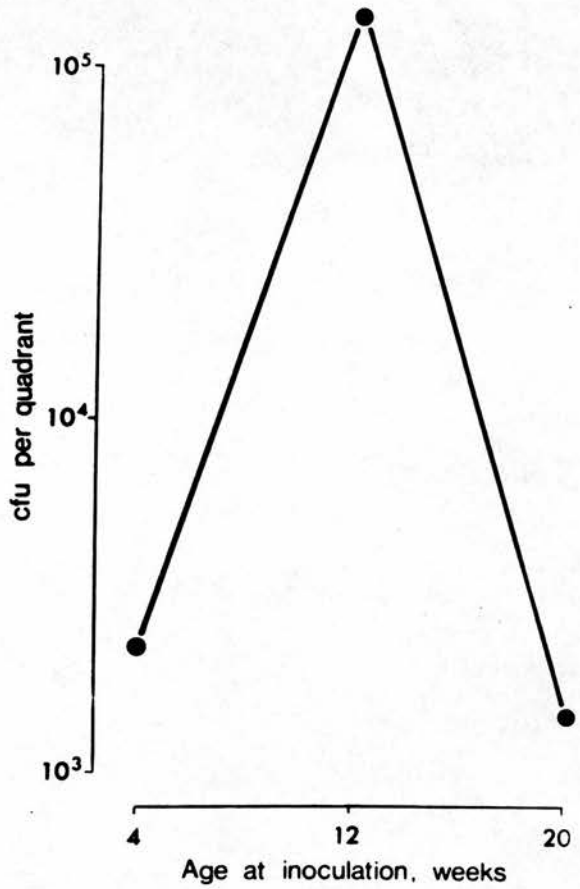


Figure 2.3 Recovery of A. viscosus 12 days after inoculation with 1.6×10^7 cfu on each of three successive days at different ages.

Recovery of A. viscosus was relatively low in animals inoculated at four weeks, peaked markedly in those inoculated at 12 weeks and fell again in those inoculated at 20 weeks. This result is similar to that described by Fitzgerald et al (1981) and indicates that age-related host factors influence the colonisation of A. viscosus. Brecher and van Houte (1979) reported a similar effect in rats infected with A. viscosus, and the phenomenon has also been described for other bacterial species (van Houte et al, 1977). The reasons for the age effect are obscure but may involve previous colonisation of the tooth surface with bacteria that can compete for the same site as A. viscosus. However, whichever the cause, this result underlined the need in subsequent experiments to use mice of the same age.

2.4 MAIN EXPERIMENTAL SCHEDULE

The experimental schedule described here and summarised in Figure 2.4 formed the basis of all the disease induction studies for different mouse genotypes. It was developed from the work of Fitzgerald et al (1981) and the results of the preliminary experiment.

For each genotype examined, a maximum of 60 mice aged 4-6 weeks were divided into five groups of 12 mice each. The first group received standard laboratory diet and no oral inoculations. The second group received special diet alone. The remaining three groups all received special diet and, after one week, oral inoculations of live A. viscosus on each of three successive days at one of three dosages: 1.6×10^6 cfu, 1.6×10^7 cfu or 1.6×10^9 cfu. For the sake of brevity, these inoculation doses are designated simply as 10^6 cfu, 10^7 cfu and 10^9 cfu in all subsequent sections. All these inoculations were performed as detailed previously. After inoculation, all inoculated animals were given food and water ad libitum until the end of the experimental period 12 weeks later, when all mice were sacrificed by ether inhalation.

Two out of each group of 12 mice had their molars removed by quadrant for A. viscosus recovery as described previously. For the remaining 10 mice per group, the following procedures were carried out:

(a) Immediately post mortem, blood was aspirated from the inferior vena cava and samples from the 10 mice of each group were pooled. The resultant serum was then stored at -60°C for antibody studies.

(b) The spleens and mesenteric lymph nodes were removed and pooled by group to provide sufficient numbers of cells for cellular immunological studies.

(c) The jaws of each mouse were dissected out and stored in separately labelled bottles of buffered formol saline. The right hemimandibles were subsequently defleshed and assessed for bone loss, while the left hemimandibles were retained for routine histological study.

Details of these methods are given in subsequent chapters.

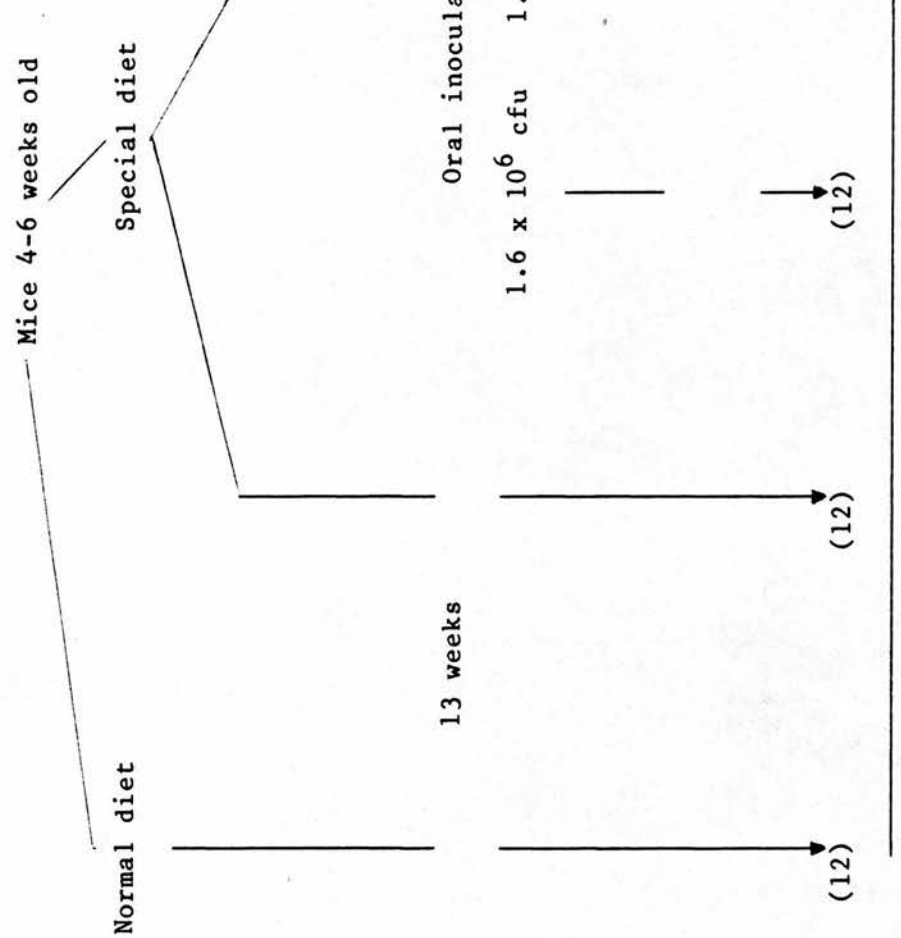


Figure 2.4 Diagrammatic representation of the schedule used in the main experiment.

2.5 STATISTICAL ANALYSIS

All measurements were transferred to computer data files and analysed using the following routines from SPSSx (SPSS Inc, 1983): FREQUENCIES, CROSSTABS, BREAKDOWN, ONEWAY, ANOVA, MANOVA and NONPAR CORR.

Results are expressed in tabular or graphical form as appropriate.

C H A P T E R 3

BONE LOSS

3.1 PREVIOUS METHODS OF ASSESSMENT

Various means of assessing periodontal disease experience in animals from gross jaw specimens have been used. Some of these are summarised below. All methods have been applied to defleshed specimens.

(a) Method of Keyes & Gold (1955): The distance from the amelocemental junction (ACJ) to the adjacent alveolar bone crest was recorded for both the maxilla and mandible of Syrian hamsters. Measurements were made both buccally and lingually at sites corresponding to the positions of the molar cusps, and were recorded to the nearest 0.2 mm using a dissecting microscope and a graduated metal blade. The total count of 0.2 mm units at all sites in the molar region was used as a means of expressing periodontal destruction.

(b) Method of Gupta & Shaw (1956b): The degree of alveolar resorption in both jaws of rice rats was expressed in terms of the ACJ to alveolar crest distance at sites as used by Keyes and Gold (1955), but in terms of arbitrary categories:

0 = No detectable bone resorption

1+ = Minor alveolar resorption with $\frac{1}{4}$ of the root exposed

2+ = Appreciable bone loss with $\frac{1}{4}$ to $\frac{1}{2}$ of the root exposed

3+ = Moderate bone loss with $\frac{1}{2}$ to $\frac{3}{4}$ of the root exposed

4+ = Severe bone loss with essentially no remaining alveolar support for the root

(c) Method of Gustafson (1969): The distance between the ACJ and alveolar bone crest was measured in mink at five different sites with a probe graduated at 0.2 mm intervals. The sites were:-

(i) Buccal : distal surfaces of two premolars

(ii) Lingual: distal surfaces of 1st molar

(iii) Bifurcation of 1st molar root

(iv) Mesial part of bifurcation of 2nd molar root

(d) Method of Lavine et al (1976): The distance between the ACJ and alveolar bone crest on the buccal side only was measured in mink at the following sites:-

(i) Midbuccal and distobuccal of 1st premolars

(ii) Midbuccal only of maxillary 2nd premolar

(iii) Mesial and distal of mandibular 2nd premolar

(iv) Mesial and distal of mandibular 3rd premolar.

(e) Method of Messer et al (1982): The lingual aspect of mouse right hemimandibles was lightly stained with 'Paragon' stain to emphasise the ACJ. The distance from the ACJ to the alveolar crest was measured for each root of each molar using a dissecting microscope incorporating a micrometer scale. The length of root exposed was then expressed as a percentage of the total root length based on the average measurement obtained for each root length from 30 extracted molars. Results were expressed either as a mean score for all teeth or for individual roots.

Other variations on the theme exist, but in the current study it was decided to introduce a slightly different form of measurement, by which the whole area of exposed molar roots, on lingual view of defleshed hemimandibles, was recorded. This had the advantage of being a simple way of providing a single bone loss score for each animal which did not rely on arbitrary scores given after viewing specimens or on measurements made only at specific designated points. It was thus hoped that the technique,

details of which are described in Section 3.2, would provide a more accurate measure of periodontal bone destruction.

3.2 METHOD OF ASSESSMENT USED IN THE THE PRESENT STUDY

After sacrifice, the jaws were dissected out and the right hemimandibles defleshed by placing them in individually labelled water-filled boiling tubes which were then boiled for 10 minutes. After boiling, the water was replaced by a 5% solution of papain in isotonic saline and the tubes placed in a 37°C incubator for 48 hours. The method of defleshing was taken from Luther (1949). The jaws were then removed, rinsed in clean cold water, carefully brushed to remove tissue fragments, air dried at room temperature and subsequently placed in van Gieson stain for approximately 10 minutes. Stain was taken up by cementum and bone but not by enamel, thus throwing the ACJ into sharp relief. The lingual side of the stained specimens was then viewed via a dissecting microscope (Zeiss Ltd, West Germany) incorporating a grid graticule composed of 0.25 mm sided squares (Graticule Ltd, Kent, UK), at a fixed magnification of x25. At this magnification, the image was divided into squares, the sides of which were equivalent to 0.01 mm on the object. The area of exposed molar root surface was measured by counting the number of squares bounded by the ACJ, the lingual alveolar bone crest, the anterior border of the anterior first molar root and the posterior border of the third molar root, as illustrated in Figure 3.1.

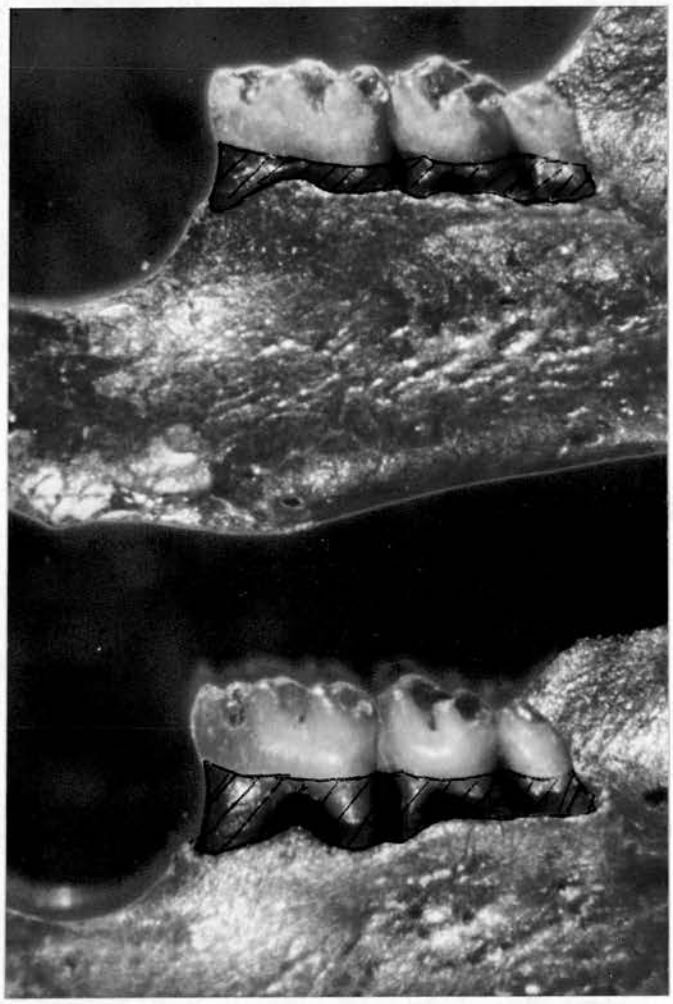


Figure 3.1 Lingual view of defleshed right hemimandible showing the area of exposed molar roots that was measured. ▨

3.3 NATURALLY OCCURRING BONE LOSS

Naturally occurring bone loss, as a consequence of the normal oral flora, standard laboratory diet and advancing age, was examined in bg/bg mice and non-mutant control C57 mice to assess the range of periodontal disease experience that might be expected for animals in which no active attempt had been made to induce disease. The results are shown in Table 3.1. Analysis of variance by genotype, age and sex for these groups of mice is summarised in Table 3.2. There was a significant difference between genotypes (with bg/bg mice showing more bone loss at all ages), a significant age effect (with bone loss increasing with age in both genotypes) but no overall difference between the sexes. There were, however, significant interactions between genotype and age, genotype and sex, age and sex and genotype, age and sex. The nature of these interactions is illustrated in Figure 3.2. The age effect was more pronounced in bg/bg mice than C57 controls, bone loss tended to be greater in beige females than males but less in C57 females than males, and the difference between the sexes was most prounounced in the oldest age group of bg/bg mice.

The increase in bone loss with age was consistent with the physiological age changes described previously (1.2.3), and the higher levels of bone loss in bg/bg mice, and more rapid progression with age, were anticipated in view of this mutant's defects of immune function. However, the massive periodontal destruction described in the Chediak-Higashi mink (Lavine et al, 1976) was not found. The reasons for the genotype by sex interaction shown in Table 3.2 and Figure 3.2 are unclear.

Table 3.1 Mean bone loss (mm^2) in beige mice and non-mutant C57 controls at different ages. n = number of mice. Sexes combined.

		Age in weeks			
		6	21	36	52
C57	n	10	9	9	10
	Mean	0.634	0.729	0.871	0.942
	s.e.	0.009	0.009	0.011	0.007
bg/bg	n	10	10	10	9
	Mean	0.690	0.846	0.908	1.246
	s.e.	0.012	0.009	0.022	0.041

Table 3.2 Analysis of variance for bone loss by genotype (bg/bg versus C57), age and sex in uninoculated mice.

Source of variation	ss	DF	ms	F	Significance
Main effects					
Genotype	0.308	1	0.308	124.1	0.000
Age	1.887	3	0.629	253.1	0.000
Sex	0.006	1	0.006	2.5	0.122
2-way interactions					
Genotype/Age	0.201	3	0.067	27.0	0.000
Genotype/Sex	0.011	1	0.011	4.6	0.036
Age/Sex	0.024	3	0.008	3.2	0.030
3-way interactions					
Genotype/Age/Sex	0.023	3	0.008	3.1	0.032
Residual	0.151	61	0.002		
Total	2.611	76	0.034		

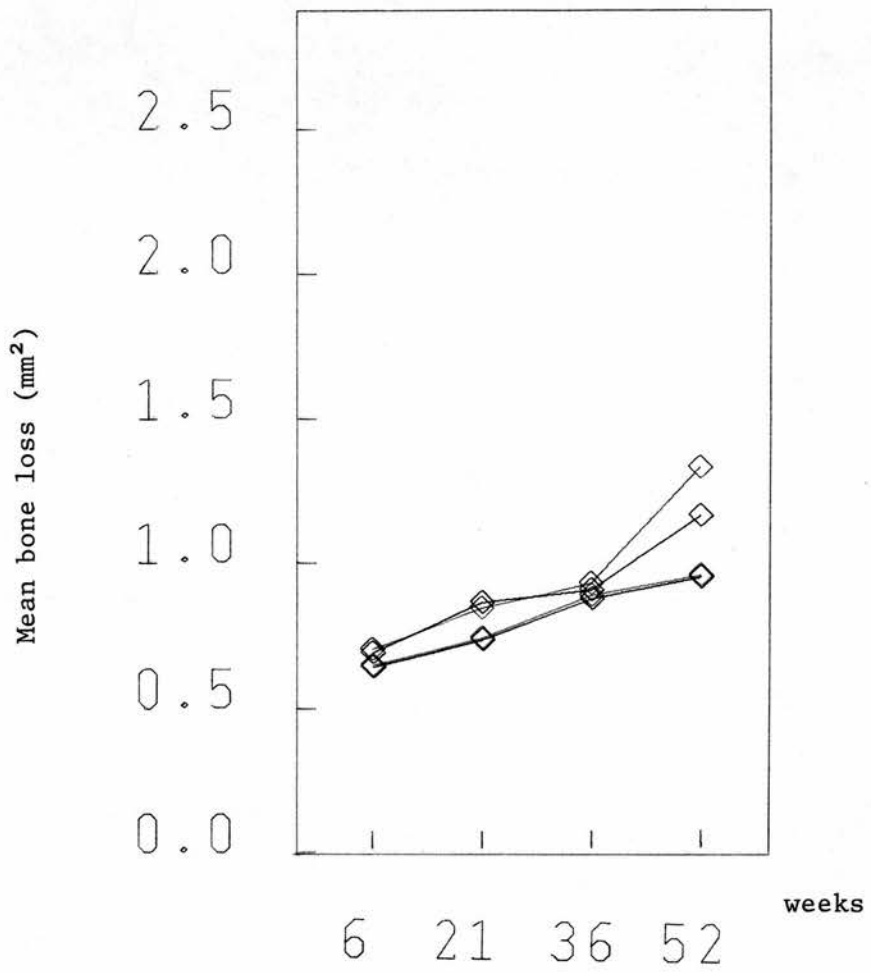


Fig 3.2 Bone loss in male and female beige mice and non-mutant C57 controls at different ages.
C57 female (blue), C57 male (red),
bg/bg female (black), bg/bg male (green).

3.4 INDUCED BONE LOSS

3.4.1 SIX WEEKS AFTER INOCULATION

Bone loss six weeks after inoculation was assessed to see (a) whether bone loss could be related to inoculum size and (b) how the absolute levels of destruction achieved in this time compared with those found 12 weeks after inoculation. This experiment was performed only in BALB/c mice and the results are summarised in Table 3.3. Uninoculated controls and all three inoculation groups showed significantly greater bone loss six weeks after inoculation than uninoculated controls at the start of the experiment. Mice inoculated with 10^7 cfu showed significantly greater bone loss than the other three groups at the same age, but neither of the other two inoculation groups was significantly different from uninoculated controls at the same age.

Table 3.3 Mean bone loss (mm^2) in BALB/c mice at age 7 weeks and 6 weeks after inoculation (age 13 weeks). n = number of mice.

DIET:		NORMAL	SPECIAL	SPECIAL	SPECIAL
INOCULUM (cfu):		0	10^6	10^7	10^9
AGE					
7 weeks	n	10			
	Mean	0.580			
	s.e.	0.020			
13 weeks	n	8	8	9	10
	Mean	0.721	0.760	0.836	0.747
	s.e.	0.020	0.014	0.028	0.012

3.4.2 TWELVE WEEKS AFTER INOCULATION

The results of all bone loss measurements made 12 weeks after inoculation, with the exception of those for a special immunisation experiment described in Chapter 9, are summarised in Table 3.4. They illustrate three basic points:

(a) The standard errors are very small relative to the means, implying that the variation within groups was small enough to estimate group means with reasonable accuracy using samples of around 10 mice per group;

(b) Differences between genotypes and inoculation groups were generally large compared with the group standard errors, so that many of these differences are statistically significant;

(c) For each genotype studied, maximal bone loss was always associated with inoculations of 10^7 cfu.

More detailed analysis is described below.

Table 3.4 Mean induced bone loss in mm^2 12 weeks after inoculation. n = number of mice.

DIET:		NORMAL	SPECIAL	SPECIAL	SPECIAL	SPECIAL
INOCULUM:		0	0	10^6	10^7	10^9
BALB/c	n	10	10	9	9	9
	Mean	0.933	0.931	0.926	1.484	0.927
	s.e.	0.030	0.011	0.036	0.024	0.040
C57	n	10	9	10	10	10
	Mean	0.652	0.648	0.646	0.982	0.728
	s.e.	0.008	0.009	0.023	0.011	0.009
+/bg	n	10		10	10	10
	Mean	0.661		0.690	1.010	0.730
	s.e.	0.020		0.023	0.023	0.012
bg/bg	n	10		9	9	9
	Mean	0.742		0.757	0.996	0.801
	s.e.	0.012		0.017	0.014	0.011
ob/ob	n	8			8	8
	Mean	0.628			0.894	0.722
	s.e.	0.011			0.013	0.009
CBA	n	5		5	5	5
	Mean	0.796		0.800	1.212	0.812
	s.e.	0.012		0.008	0.009	0.009
+/xid	n	5		5	5	5
	Mean	0.790		0.800	1.206	0.810
	s.e.	0.010		0.007	0.011	0.007
xid/Y	n	5		4	5	5
	Mean	0.704		0.702	1.362	0.710
	s.e.	0.008		0.010	0.009	0.007

3.4.2.1 Special diet alone

The effect of special diet as opposed to standard laboratory diet was analysed in uninoculated mice using analysis of variance by diet and by sex for the BALB/c and C57 strains separately (Table 3.5). Neither diet nor sex showed a significant association with bone loss in these strains and there was no diet by sex interaction. The results indicate that induced bone loss was dependent on the combined effect of special diet and inoculated A. viscosus and that any difference between the sexes observed in inoculated animals could not be attributed to the special diet alone.

Table 3.5 Analysis of variance for bone loss by diet and sex in uninoculated BALB/c and C57 mice.

BALB/c

Source of variation	ss	DF	ms	F	Significance
Main effects					
Diet	0.000	1	0.000	0.004	0.950
Sex	0.001	1	0.001	0.102	0.754
2-way interactions					
Diet/Sex	0.014	1	0.014	2.749	0.117
Residual	0.079	16	0.005		
Total	0.094	19	0.005		

C57

Source of variation	ss	DF	ms	F	Significance
Main effects					
Diet	0.000	1	0.000	0.159	0.695
Sex	0.001	1	0.001	1.411	0.253
2-way interactions					
Diet/Sex	0.000	1	0.000	0.349	0.565
Residual	0.011	15	0.001		
Total	0.012	18	0.001		

3.4.2.2 Inbred strains

Analysis of variance for the three inbred strains by strain and inoculum size (Table 3.6) showed statistically significant differences between strains and inoculation groups as well as a significant strain by inoculum interaction. It was not possible to test for a sex effect in this comparison as CBA was represented by males only. Statistically significant differences between strain/inoculum groups from one-way analysis of variance are shown in Table 3.7.

BALB/c showed greatest bone loss, CBA intermediate bone loss and C57 least bone loss overall. Within strains 10^6 cfu did not increase bone loss above the control level, while 10^9 cfu resulted in only a relatively small effect and only in C57 mice. By contrast, 10^7 cfu produced a marked increase in bone loss in all three strains, the absolute value of the increase being greatest for BALB/c and least for C57. This was the main source of the significant strain by inoculum interaction (Table 3.6). The relative increase in bone loss however was more similar between strains, with the 10^7 cfu groups for C57, CBA and BALB/c showing respectively 1.51, 1.52 and 1.59 times the bone loss of their corresponding uninoculated controls.

Table 3.6 Analysis of variance for bone loss by strain and inoculum size.

Source of variation	ss	DF	ms	F	Significance
Main effects					
Strain	1.905	2	0.953	219.5	0.000
Inoculum	3.236	3	1.079	248.6	0.000
2-way interactions					
Strain/Inoculum	0.245	6	0.041	9.4	0.000
Residual	0.369	85	0.004		
Total	5.755	96	0.060		

Table 3.7 Summary of statistically significant differences for bone loss (mm^2) between strain/inoculum groups.
BAL = BALB/c, * $P < 0.05$.

	Genotype:	C57	C57	C57	CBA	CBA	CBA	BAL	BAL	BAL	C57	CBA	BAL
	Inoculum:	10^6	0	10^9	0	10^6	10^9	10^6	10^9	0	10^7	10^7	10^7
Mean	Geno. Inoc.												
0.646	C57 10^6												
0.652	C57 0												
0.728	C57 10^9	*	*										
0.796	CBA 0	*	*										
0.800	CBA 10^6	*	*	*									
0.812	CBA 10^9	*	*	*									
0.926	BAL 10^6	*	*	*	*	*	*						
0.927	BAL 10^9	*	*	*	*	*	*						
0.933	BAL 0	*	*	*	*	*	*						
0.982	C57 10^7	*	*	*	*	*	*						
1.212	CBA 10^7	*	*	*	*	*	*	*	*	*	*		
1.484	BAL 10^7	*	*	*	*	*	*	*	*	*	*	*	

3.4.2.3 Beige

Analysis of variance for bg/bg, +/bg and non-mutant control C57 mice by genotype, inoculum size and sex (Table 3.8), disclosed statistically significant differences between genotypes, between inoculum groups and between the sexes. There were also significant interactions between genotype and inoculum, genotype and sex and inoculum and sex. Statistically significant differences between genotype/inoculum groups from one-way analysis of variance are summarised in Table 3.9. Overall, C57 mice showed least bone loss, +/bg intermediate bone loss and bg/bg most bone loss, indicating that the beige allele was not fully recessive with respect to periodontal destruction. At 10^7 cfu, bg/bg and +/bg were not significantly different from each other indicating that, for this inoculum size, the beige allele was fully dominant with respect to induced periodontal destruction. This association between inoculum size and the difference between bone loss in bg/bg and +/bg mice contributed to the significant genotype by inoculum interaction shown in Table 3.8.

For each genotype, maximum bone loss was associated with 10^7 cfu inocula, while inocula of 10^6 cfu did not significantly increase bone loss over that found in uninoculated controls. In all three genotypes 10^9 cfu resulted in significantly more bone loss than that shown in uninoculated controls. The bone loss associated with 10^9 cfu was also significantly more than that associated with 10^6 cfu in bg/bg and C57 mice.

The nature of the sex difference and the interactions of sex

with genotype and inoculum are illustrated in Figure 3.3. In both bg/bg and +/bg animals, females showed more bone loss than males in uninoculated control groups and 10^6 cfu and 10^7 cfu inoculated groups. At 10^9 cfu, however, this was reversed for both genotypes with males showing more periodontal destruction. In C57 animals, bone loss was greater in males than females for all groups except 10^6 cfu which showed no sex differences. The reasons for these differences are not known.

Table 3.8 Analysis of variance for bone loss by genotype (bg/bg, +/bg and non-mutant C57), inoculum size and sex.

Source of variation	ss	DF	ms	F	Significance
Main effects					
Genotype	0.107	2	0.054	31.4	0.000
Inoculum	1.862	3	0.621	363.3	0.000
Sex	0.017	1	0.017	9.9	0.002
2-way interactions					
Genotype/Inoculum	0.036	6	0.006	3.5	0.004
Genotype/Sex	0.021	2	0.010	6.0	0.003
Inoculum/Sex	0.018	3	0.006	3.5	0.019
3-way interactions					
Genotype/Inoculum/ Sex	0.020	6	0.003	1.9	0.083
Residual	0.159	93	0.002		
Total	2.240	116	0.019		

Table 3.9 Summary of statistically significant differences for bone loss (mm^2) between genotype/inoculum groups for bg/bg, +/bg and non-mutant C57 control mice.
* = $P < 0.05$

	Genotype:	C57	C57	+/bg	+/bg	C57	+/bg	bg/bg	bg/bg	bg/bg	C57	bg/bg	+/bg
	Inoculum:	10^6	0	0	10^6	10^9	10^9	0	10^6	10^9	10^7	10^7	10^7
Mean	Geno. Inoc.												
0.646	C57 10^6												
0.652	C57 0												
0.661	+/bg 0												
0.690	+/bg 10^6	*											
0.728	C57 10^9	*	*	*									
0.730	+/bg 10^9	*	*	*									
0.742	bg/bg 0	*	*	*	*								
0.757	bg/bg 10^6	*	*	*	*								
0.801	bg/bg 10^9	*	*	*	*	*	*	*	*				
0.982	C57 10^7	*	*	*	*	*	*	*	*	*			
0.996	bg/bg 10^7	*	*	*	*	*	*	*	*	*			
1.010	+/bg 10^7	*	*	*	*	*	*	*	*	*			

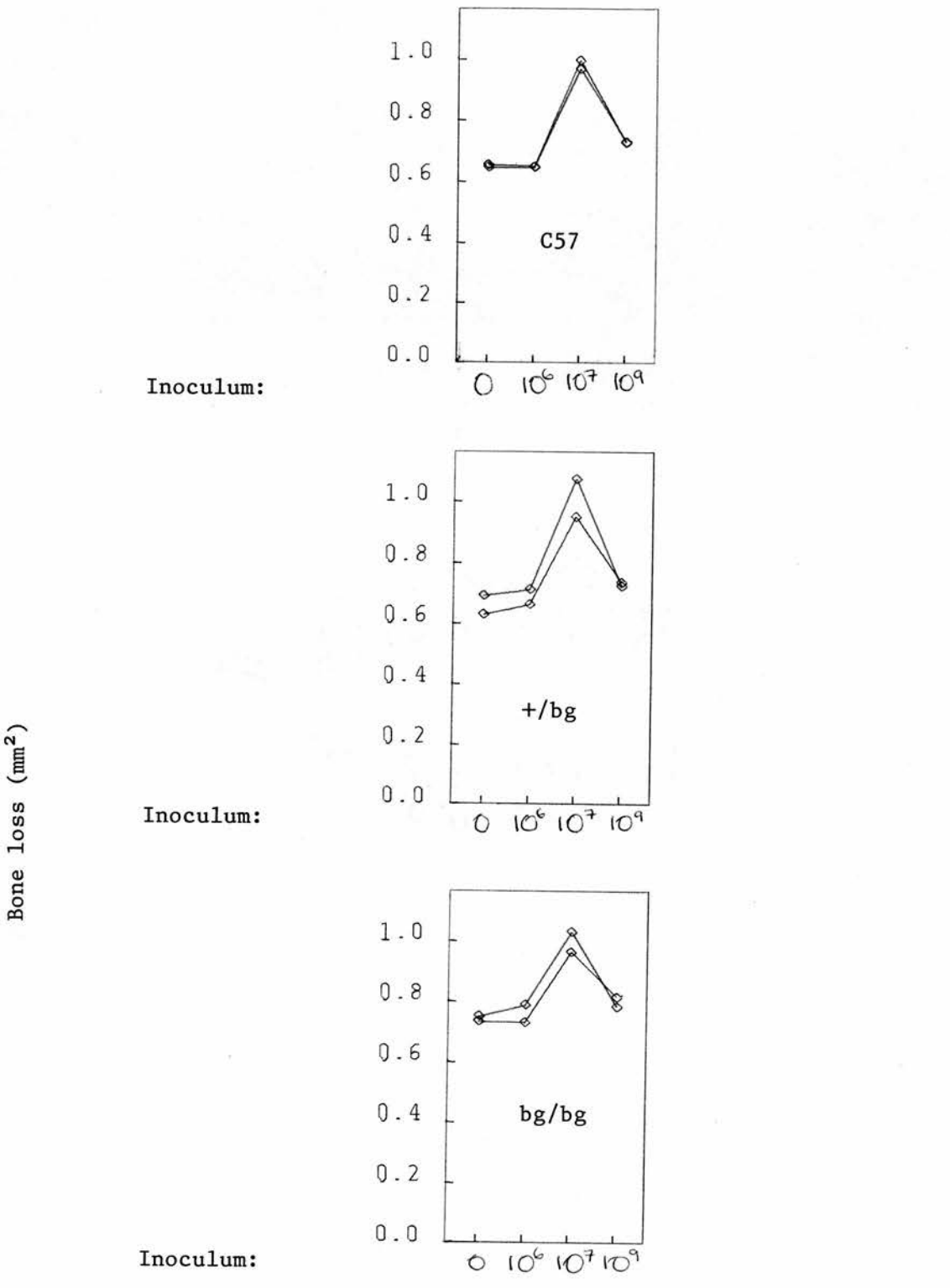


Fig 3.3 Bone loss (mm^2) in C57, +/bg and bg/bg male and female mice 12 weeks after inoculation.
Male (black), female (red).

3.4.2.4 Obese

Analysis of variance for ob/ob and non-mutant control C57 mice by genotype, inoculum size and sex showed statistically significant differences between genotypes and between inocula, but not between the sexes. The only significant interaction was that between genotype and inoculum size (Table 3.10). Table 3.11 summarises the statistically significant differences between genotype/inoculum groups from one-way analysis of variance. Contrary to expectation, bone loss was less in ob/ob mice than in C57 controls for all inoculum sizes and for uninoculated controls, although this difference was statistically significant only at 10^7 cfu, which presumably contributed to the genotype by inoculum interaction. For each genotype, maximum bone loss was again associated with 10^7 cfu, and this was significantly greater than in either the uninoculated or 10^9 cfu groups. Bone loss associated with 10^9 cfu was also significantly greater than in uninoculated controls. These results imply that homozygosity for the obese allele does not alter susceptibility to bone loss compared with C57 controls, either for naturally occurring loss or that induced by 10^9cfu. At 10^7 cfu, however, ob/ob mice appear to be relatively resistant to induced disease.

Table 3.10 Analysis of variance for bone loss by genotype (ob/ob and and non-mutant C57), inoculum size and sex.

Source of variation	ss	DF	ms	F	Significance
Main effects					
Genotype	0.020	1	0.020	20.8	0.000
Inoculum	0.873	2	0.436	447.8	0.000
Sex	0.002	1	0.002	2.3	0.140
2-way interactions					
Genotype/Inoculum	0.017	2	0.009	8.7	0.001
Genotype/Sex	0.000	1	0.000	0.0	0.844
Inoculum/Sex	0.000	2	0.000	0.1	0.891
3-way interactions					
Genotype/Inoculum/ Sex	0.001	2	0.001	0.7	0.503
Residual	0.041	42	0.001		
Total	0.954	53	0.018		

Table 3.11 Summary of statistically significant differences for bone loss (mm^2) between genotype/inoculum groups for ob/ob and non-mutant C57 control mice.
* = $P < 0.05$.

	Genotype:	ob/ob	C57	ob/ob	C57	ob/ob	C57
	Inoculum:	0	0	10^9	10^9	10^7	10^7
Mean	Geno. Inoc.						
0.628	ob/ob 0						
0.652	C57 0						
0.723	ob/ob 10^9	*	*				
0.728	C57 10^9	*	*				
0.894	ob/ob 10^7	*	*	*	*		
0.982	C57 10^7	*	*	*	*	*	

3.4.2.5 X-linked immune deficiency

The results for these animals are given in Tables 3.12 and 3.13. They show that for uninoculated controls and for 10^6, 10^7 and 10^9 cfu groups, there was no statistically significant difference between +/xid females and CBA (+/Y) males, but there were significant differences between xid/Y males and +/xid females and between xid/Y and CBA (+/Y) males. However, whereas at 10^7 cfu xid/Y showed significantly greater bone loss than +/xid and CBA (+/Y) as might be expected, at 10^6 and 10^9 cfu and for uninoculated controls xid/Y showed significantly less bone loss than +/xid and CBA (+/Y) This reversal was responsible for the significant genotype by inoculum interaction shown in Table 3.12. It is consistent with relative susceptibility to bone loss conferred by the X-chromosome derived from the CBA/Ca strain in uninoculated animals, and at 10^6 and 10^9 cfu, with the xid allele assuming importance only at 10^7 cfu which, as for all other genotypes, produced maximum bone loss.

Table 3.12 Analysis of variance for bone loss by genotype (xid/Y, +/xid and non-mutant CBA) and inoculum size.

Source of variation	ss	DF	ms	F	Significance
Main effects					
Genotype	0.013	2	0.006	16	0.000
Inoculum	2.682	3	0.894	2274	0.000
2-way interactions					
Genotype/Inoculum	0.153	6	0.026	65	0.000
Residual	0.018	47	0.000		
Total	2.866	58	0.049		

Table 3.13 Summary of statistically significant differences for bone loss (mm^2) between genotype/inoculum groups for xid/Y, +/xid and non-mutant CBA control mice. * = $P < 0.05$.

	Genotype:	xid/Y	xid/Y	xid/Y	+/xid	CBA	+/xid	CBA	+/xid	CBA	+/xid	CBA	xid/Y
	Inoculum:	10^6	0	10^9	0	0	10^6	10^6	10^9	10^9	10^7	10^7	10^7
Mean	Geno. Inoc.												
0.703	xid/Y 10^6												
0.704	xid/Y 0												
0.710	xid/Y 10^9												
0.790	+/xid 0	*	*	*									
0.796	CBA 0	*	*	*									
0.800	+/xid 10^6	*	*	*									
0.800	CBA 10^6	*	*	*									
0.810	+/xid 10^9	*	*	*									
0.812	CBA 10^9	*	*	*									
1.206	+/xid 10^7	*	*	*	*	*	*	*	*	*			
1.212	CBA 10^7	*	*	*	*	*	*	*	*	*			
1.362	xid/Y 10^7	*	*	*	*	*	*	*	*	*	*	*	

3.4.3 DISCUSSION

Comparing the uninoculated BALB/c mice aged seven weeks and 13 weeks in Table 3.3, it is apparent again that bone loss increases with time as a natural phenomenon. Comparing the inoculated BALB/c mice aged 13 weeks with their uninoculated controls (Table 3.3) shows that, even six weeks post-inoculation, there was more bone loss associated with 10^7 cfu than with 10^6 or 10^9 cfu. The differences between groups, however, were much smaller than those found 12 weeks after inoculation (Table 3.4), so this latter time was used exclusively in all subsequent bone loss experiments.

To analyse the bone loss results further, they were standardised by using the ratio of bone loss found 12 weeks after inoculation to that found in uninoculated controls of the same age and genotype. These ratios are shown in Figure 3.4. For each genotype, the same pattern was found with the ratio for 10^6 cfu being least, that for 10^7 cfu greatest and that for 10^9 cfu approximately down to the 10^6 cfu level. In view of the clumping of ratios around unity for 10^6 and 10^9 cfu, there is little that can be said about differences of response between genotypes. However, at 10^7 cfu, xid/Y mice showed by far the greatest ratio and bg/bg clearly the lowest.

Taking into account the neutrophil and NK cell defects of beige mice (2.1.2.1), it is logical to conclude that the functions of these cells are not important in protection against induced disease. In X-linked immune deficient mice, there are marked B-cell defects and the secretory immune system operates only at

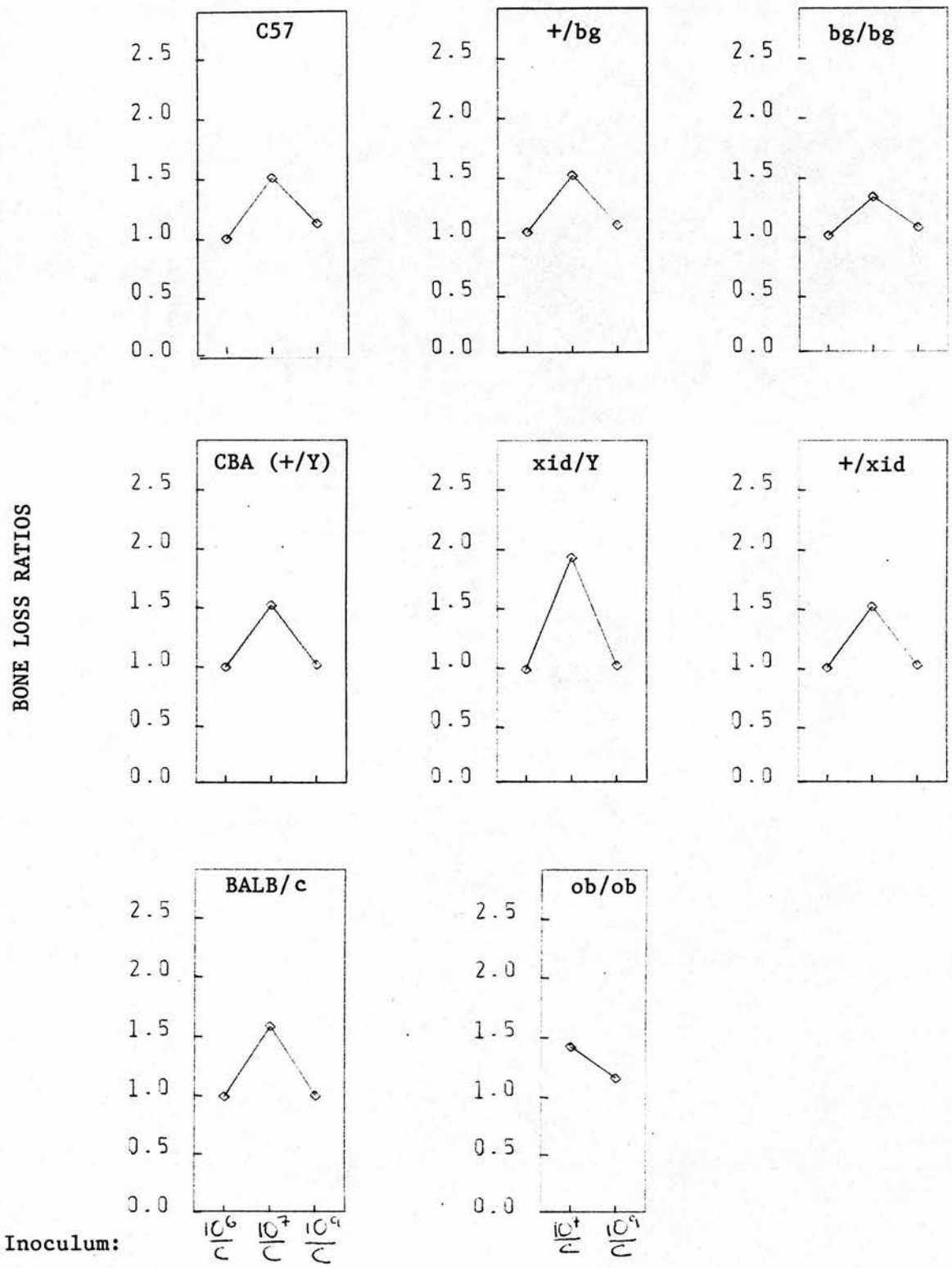


Fig 3.4 Ratio of bone loss found 12 weeks after inoculation with A. viscosus to that found in uninoculated mice of the same age and genotype (C) sexes combined.

reduced capacity (2.1.2.3). The secretory immune system would therefore seem to be significantly involved in the host response to A. viscosus.

The response of the third mutant, obese, to 10^7 cfu, was little different from that of C57 controls, therefore, any components of the immune system defective in these animals (2.1.2.2) are not important in protection against induced disease. Monitoring of polymorphonuclear leucocyte (PMN) function, the cellular immune system, and both nonspecific and specific anti-A. viscosus immunoglobulin production might help to explain the differences of response to 10^7 cfu found in these genotypes.

The fact that 10^6 cfu inoculations did not result in significantly more bone loss than controls could be ascribed to insufficient numbers of organisms to elicit the required changes in the host for bone destruction to occur. Increased bone loss associated with 10^9 cfu could be explained by assuming that this inoculum size did elicit the required host changes. The fact that 10^9 cfu produced significantly lower levels of bone loss than 10^7 cfu, and that these were sometimes not significantly greater than those in controls, suggests the action of some immunological mechanism, primed by inoculum size, which influenced the degree to which destruction occurred. One such possibility is tolerance. This phenomenon is best explained as follows.

Normally, administration of a foreign antigen to an animal results in an antibody response. An antigen, however, does not always stimulate detectable antibody and, in certain circumstances, highly immunogenic antigens can produce a state of unresponsiveness which is permanent. This unresponsiveness is specific for the initiating antigen and administration of further

doses does not break the unresponsive state. This non-response is known as tolerance. The most obvious example is the absence of an immune response to self-antigens in the immunologically mature adult. In some manner, the developing lymphoid system learns that the antigens encountered in fetal life and in the early stages of development are self-antigens. There appears to be some form of genetic control over immune responses, the net effect of which is the termination of any 'self-directed' immunocompetent cell line, or at least of its effects. In experimental animals, tolerance can be induced by giving large doses of antigen (1 mg g^{-1} body weight). This 'high zone tolerance' is dependent on T and B cell unresponsiveness and is usually transient. Injections of less than 1 mg g^{-1} body weight can induce 'low zone tolerance', which appears to be persistent and associated with T cell unresponsiveness (Amos, 1981). Thus, in the present study, 10^6 cfu perhaps corresponded to low zone tolerance, and 10^9 cfu to high zone tolerance. Investigation of T and B cell function could clarify the situation.

The route of administration of antigen is also very important. Using oral administration of bovine serum albumin (BSA) to rats, Thomas and Parrott (1974) showed that tolerance could be induced by that route. These authors suggested that, although large quantities of BSA were administered orally, most would be digested and the amount absorbed would thus be small. They suggested that, in this case, the tolerance induced was low zone tolerance.

One main problem with the oral route of administration as used in the current work is the question of whether the swallowed

macromolecules retain antigenic properties after digestion. Rothberg (1969) demonstrated that, after 'digestion' of BSA with pepsin, a moiety remains which retains the molecular weight and the antigenicity of native BSA. Crumpton (1967) and Atassi and Saplin (1968) showed that proteolytic digestion of large molecules can result in products which share antigenic determinants with the native antigen. Therefore, the induction of tolerance via the oral route could be due to absorption of either small quantities of the native protein, or of fragments of the protein bearing antigenic determinants in common with the native protein. The latter possibility is particularly attractive in view of the demonstration by Ada and Parish (1968) that digestion products of flagellin are toleragenic in adult rats, while the whole molecule is strongly immunogenic. Thus, it can be assumed that some antigenic determinants are left on the A. viscosus organisms after ingestion and digestion.

Oral or intragastric administration of antigens is known to cause the appearance of circulating antibodies which frequently are predominantly of the IgA class (Heremans, 1974). Some of this antibody is made locally in the immunised gut wall (Vaerman & Heremans, 1970; Vaerman et al, 1973), but an additional fraction originates in extraintestinal lymphoid tissues, particularly the spleen. Andre et al (1975) found that if mice were given a four-day course of intragastric placement of sheep red blood cells, specific antibodies, mainly of the IgA class, were produced. However, if the dosage was repeated 2-3 weeks later, there was a complete absence of response and it took several

months for systemic reactivity to renewed intragastric challenge to be restored to the level of the primary response (Andre et al, 1973). Reports from different authors (Andre et al, 1974; Walker et al, 1972, 1973) indicate that immunisation by the digestive route leads to impaired enteric absorption of macromolecular antigens. Andre et al (1974) showed that rats which received a single dose of 200 mg human serum albumin by gastric intubation had a greater than 50% reduction in capacity of the gut to absorb intact albumin and that secretory IgA present in the enteric lumen at that time was responsible for the effect. This led to the suggestion that it was reduced absorption not tolerance that was induced in animals given oral antigens.

However, Andre et al (1975) showed that orally immunised animals did not respond to rechallenge with the same antigens by the digestive route or by injection of the challenging dose intraperitoneally, thus clearly demonstrating that there was true tolerance. In addition, it was found that serum from intragastrically immunised mice contained a very active toleragen which was capable in vivo of conferring tolerance to non-immune recipient mice and in vitro of paralysing the activity of antibody producing cells (Andre et al, 1975). This toleragen seemed to consist of immune complexes with IgA as the antibody. These authors concluded that such a mucosal type of immune response takes place in the gut wall itself and that, to some extent, it may extend to distant lymphoid tissues such as the spleen. They suggested that the portal lymphoid tissue of the liver also be considered as it is in direct continuity with the lamina propria

of the gut and, like the latter, is a preferred site of IgA production (Dive et al, 1974). In addition, it is exposed directly to enterically absorbed antigen carried by the mesenteric venous blood. Thus, if the mechanism of tolerance via IgA immune complexes is correct, this would explain systemic hyporesponsiveness by antigen given orally (Chase, 1946; Cantor & Dumont, 1967) or injected into the portal circulation (Battisto & Miller, 1962; Mayer et al, 1965), as well as the suppression of these effects when the liver is bypassed by a portocaval transposition (Cantor & Dumont, 1967). It has been suggested that IgA antibody-ingested antigen complexes might persist for a long time. However, Swarbrick et al (1976) demonstrated that passive transfer of IgA antibodies promotes rapid elimination of antigen and increases rather than reduces the antibody response to subsequent immunisation.

The form in which antigen is administered appears to be important also. Cox & Taubman (1984), studying salivary IgA production, found the response to be greater if the antigen was in particulate rather than soluble form. In the current work whole bacteria were used, and it was hoped that this would lead to good immunoglobulin responses.

Conclusions based on bone loss data therefore confirmed the need in the present study for investigations of PMN function and both humoral and cellular components of the immune system. The findings emphasised the probable relevance of the secretory immune system in general and of IgA levels in particular, and the need to study cells from both spleens and mesenteric lymph nodes

to elucidate the inoculum size dependent response. The hypothesis that 10^6 cfu induced low zone tolerance and 10^9 cfu induced high zone tolerance is considered again in subsequent chapters in the light of results of the immunological experiments.

C H A P T E R 4

HISTOLOGY

4.1 INTRODUCTION

Histology has been used by a number of investigators as an indicator of both the presence and severity of natural and experimental periodontal disease in different mouse genotypes. Examples include leukaemic mice (Brown et al, 1969; Flanagan et al, 1970), pocket mice (Shklar & Person, 1975), albino mice (Gilmore & Glickman, 1959) and diabetic mice (Elgeneidy et al, 1974). Many of the histological features seen in periodontitis in animals have been assessed in attempts to measure the progress of the disease. The features noted in such investigations can be listed as follows:

(a) Epithelium, mucosa and submucosa
    (i) Presence or absence of keratinised layer on gingival epithelium
    (ii) Epithelial proliferation, both site and degree
    (iii) Apical growth of epithelial attachment

(b) Periodontal membrane
    (i) Changes in orientation of periodontal fibres or signs of their degeneration
    (ii) Increased thickness of periodontal membrane
    (iii) Number and size of blood vessels

(c) Bone
    (i) Presence of either localised or generalised resorption
    (ii) Whether bone loss is horizontal or vertical
    (iii) Position of ACJ relative to bone crest
    (iv) Number and distribution of bone incremental lines
    (v) Relative numbers of osteoblasts and osteoclasts

(d) Cement

(i) Signs of cement resorption

(ii) Extent and distribution of secondary cement formation

(iii) Number and distribution of cemental incremental lines

(e) Tooth

(i) Presence or absence of occlusal abrasion

(ii) Presence or absence of root cavitation

(f) Inflammation

Position, degree and nature of any inflammatory infiltrate

(g) Plaque/calculus

Presence and position of plaque/calculus

(h) Foreign bodies

Presence of hair or food impaction

Examination of most of these features was attempted for all genotypes used in the current study.

As already discussed (1.2.3), it is important when studying murine periodontal disease that age changes are not misinterpreted as disease. In this context, Gilmore and Glickman (1959) showed that cement thickness increases with age in the mouse so that the increasing distance between ACJ and alveolar crest is not wholly pathological in origin. These authors also noted that the gingival

wall of the gingival sulcus is lined by keratinised oral epithelium which extends below the level of the base of the sulcus where it lies lateral to and is contiguous with the enamel epithelium. At all stages, the base of the sulcus is located at the most coronal level of the enamel epithelium (see Figure 4.1). With age, however, the most coronal portion of the enamel epithelium undergoes progressive atrophy leading to the formation of a thin cuticle with the base of the sulcus shifting towards the ACJ. These authors also noted that some degree of inflammation was a constant finding in the connective tissue beneath the base of the sulcus, associated with irritation from food debris and calculus. The degree of inflammation was shown to increase with the age of the animal. Epithelial changes accompanied the increase in inflammation with epithelial proliferation occurring apically and laterally in the form of elongated finger-like projections. This hyperplasia was originally confined to the enamel epithelium and was predominantly apical but, as the inflammation increased, proliferation of the oral epithelium was observed also. This then led to formation of a pocket below which bone crest resorption was visible, though this was found essentially only in old animals.

The histological component of the current work was not intended to be either conclusive or exhaustive. It was included to complement the other aspects of the study and to see if it was possible to relate bone loss findings to histological manifestations of disease.

4.2 MATERIALS AND METHODS

After sacrifice, left hemimandibles were placed in individually labelled sample bottles containing neutral buffered formalin. These specimens were subsequently decalcified in formic citrate solution (see Appendix) for 14 days at room temperature, trimmed down to the molar segments and embedded in paraffin wax. Approximately sixty 5 μm sections were then cut buccolingually in the coronal place from the first molar end of each block and these were stained alternately with haematoxylin and eosin (H & E) van Gieson and azure stains (see Appendix). H & E gave a general overview of the histological appearance, azure was particularly useful for studying bone and cement, while van Gieson, which stains collagen, was of value in studying the orientation and integrity of the periodontal ligament fibres. Specimens from each genotype/inoculum group of the main study and from each of the different groups in the immunisation experiment (see Chapter 9) were examined, together with uninoculated beige mice at ages 6, 21, 36 and 52 weeks (10 mice per age group). An attempt was made to assess the features listed above, with the exception of the relative numbers of osteoblasts and osteoclasts.

4.3 R E S U L T S

No consistent pattern could be discerned relating histological appearance to either genotype or inoculation group. In some sections the base of the gingival sulcus was lying at the ACJ. This was associated with either minimal neutrophil infiltration (Figures 4.2; 4.3) or with greater numbers of these cells migrating through the epithelial attachment and adjacent connective tissue (Figures 4.4; 4.5). In other sections the epithelial attachment was lying entirely on the cementum but in no case was there evidence of dense inflammation (Figure 4.6). In only a few apparently random sections was there evidence of active bone resorption with osteoclasts (Figure 4.7). Figures 4.8 to 4.10 are of representative sections from beige mice aged 6 weeks to 52 weeks.

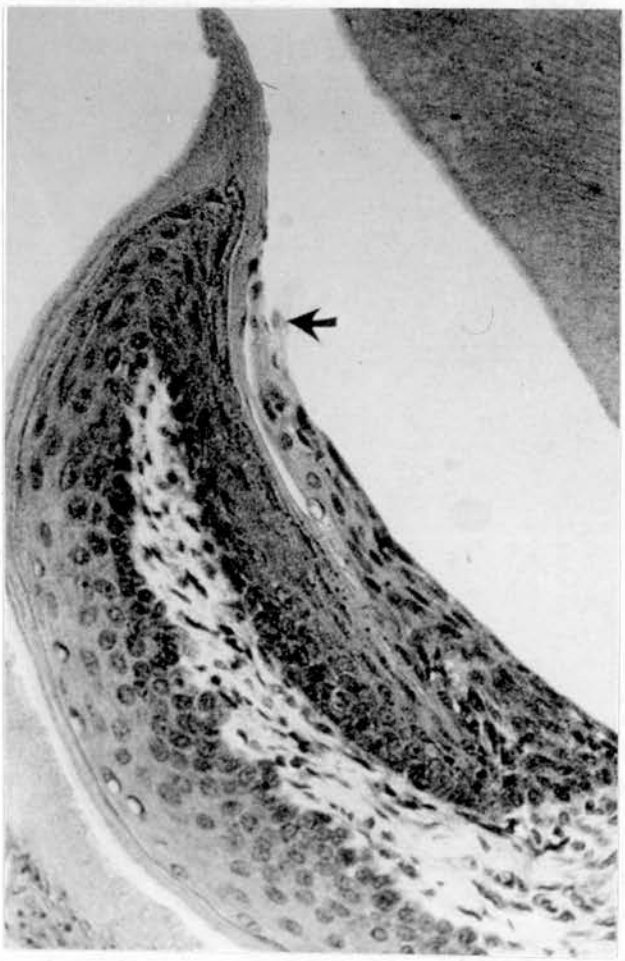


Fig 4.1 Buccolingual section through the lingual gingival sulcus in a 5 week old RAP mouse. The base of the sulcus, indicated by the arrow, is at the most coronal level of the enamel epithelium. Magnification x440. Stained H & E (reproduced from Gilmore & Glickman, 1959).

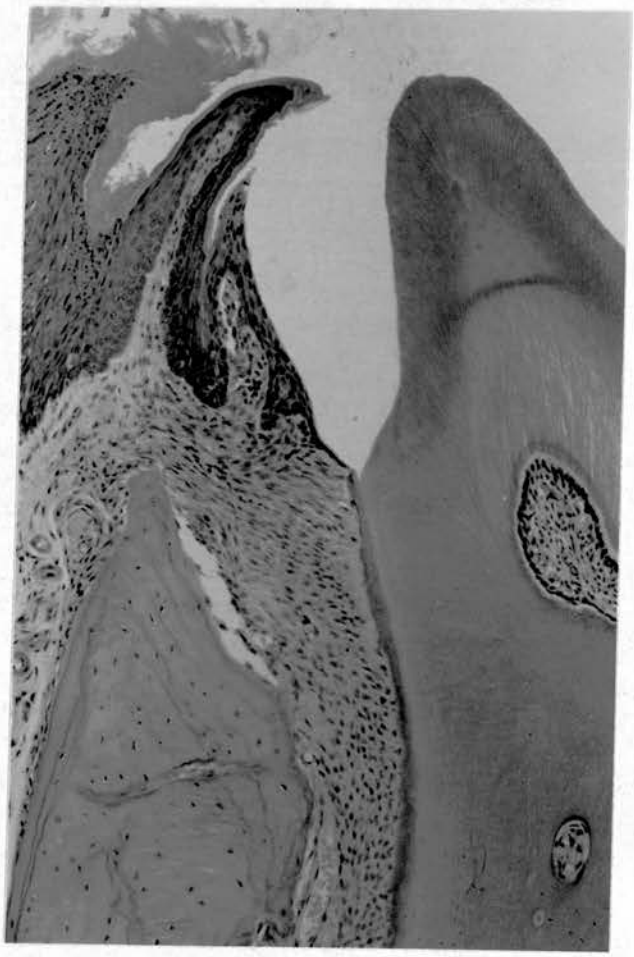


Fig 4.2 Buccolingual section through the buccal gingival sulcus in the lower left first molar region of a 10^9 cfu inoculated xid/Y mouse showing the base of the epithelial attachment located at the ACJ and associated with minimal inflammation. Magnification x120. Stained H & E.

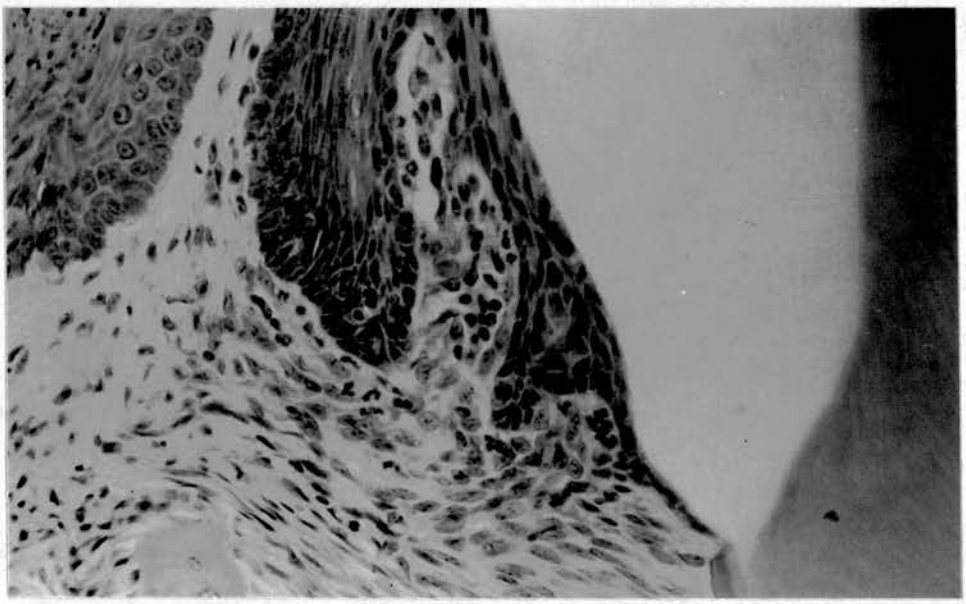


Fig 4.3 Higher power view of specimen shown in Figure 4.2.

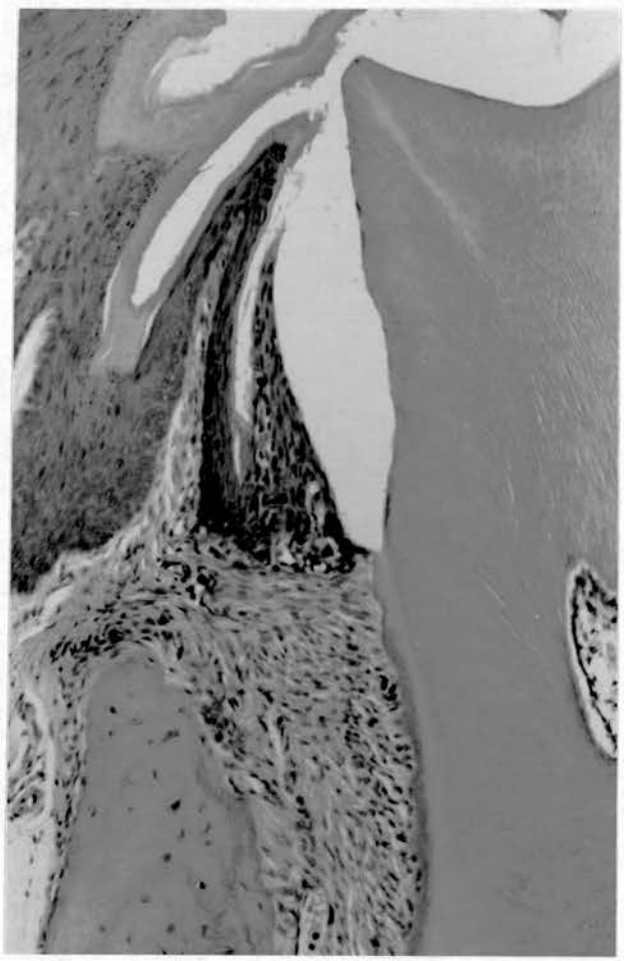


Fig 4.4 Buccolingual section through the buccal gingival sulcus in the lower left first molar region of a 10^7 cfu inoculated CBA mouse showing the base of the epithelial attachment located at the ACJ and associated with noticeable inflammation.
Magnification x120. Stained Van Gieson.

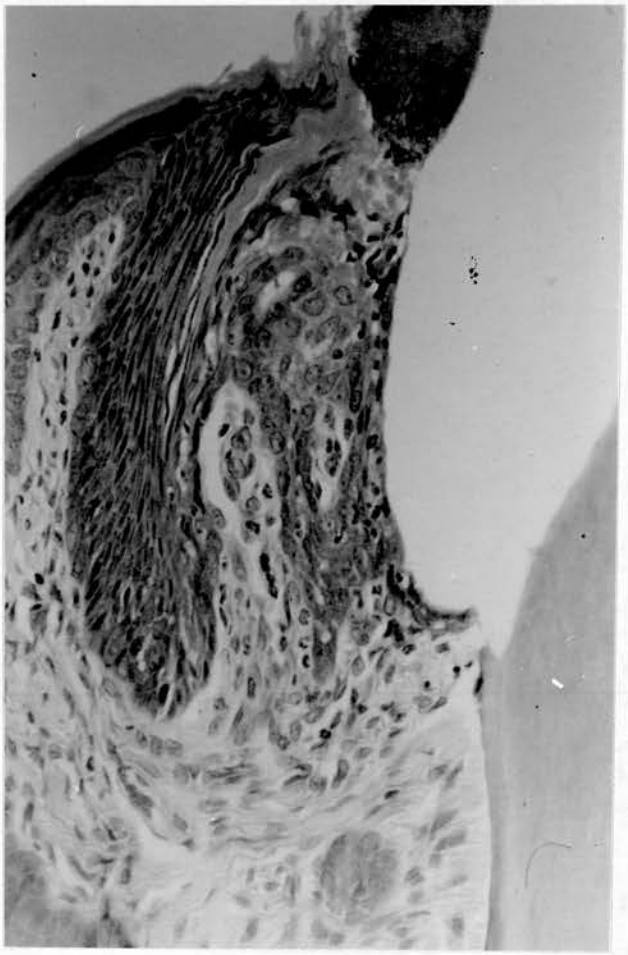


Fig 4.5 Buccolingual section through the buccal gingival sulcus in the lower left first molar region of a 10^9 cfu inoculated xid/Y mouse showing the base of the epithelial attachment located at the ACJ. Neutrophils can be seen migrating through the sulcus epithelium.
Magnification x300. Stained H & E.

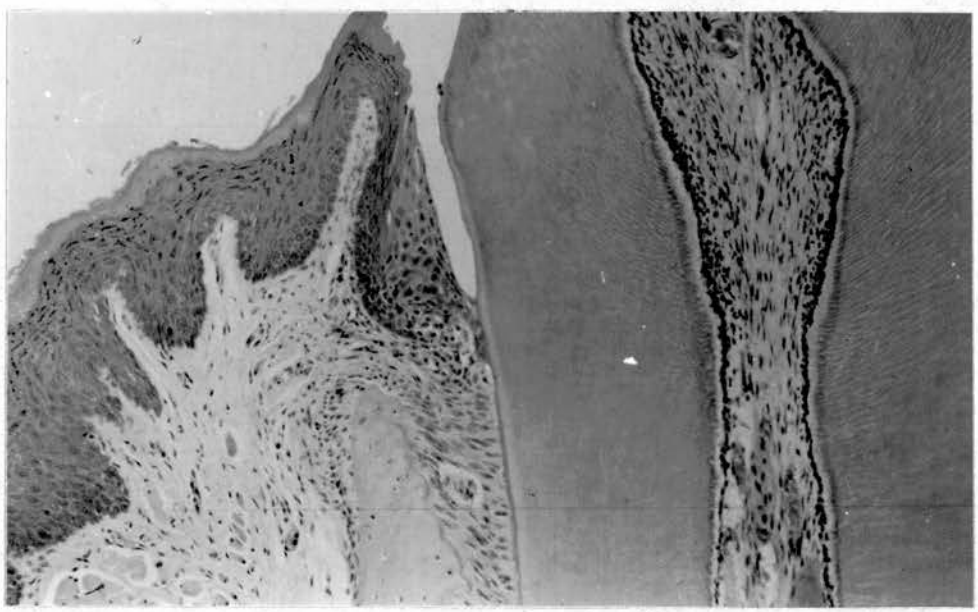


Fig 4.6 Buccolingual section through the lingual gingival sulcus in the lower left first molar region of a 10^7 cfu inoculated BALB/c mouse showing the epithelial attachment lying on cementum but without a dense inflammatory infiltrate. Magnification x120. Stained H & E.

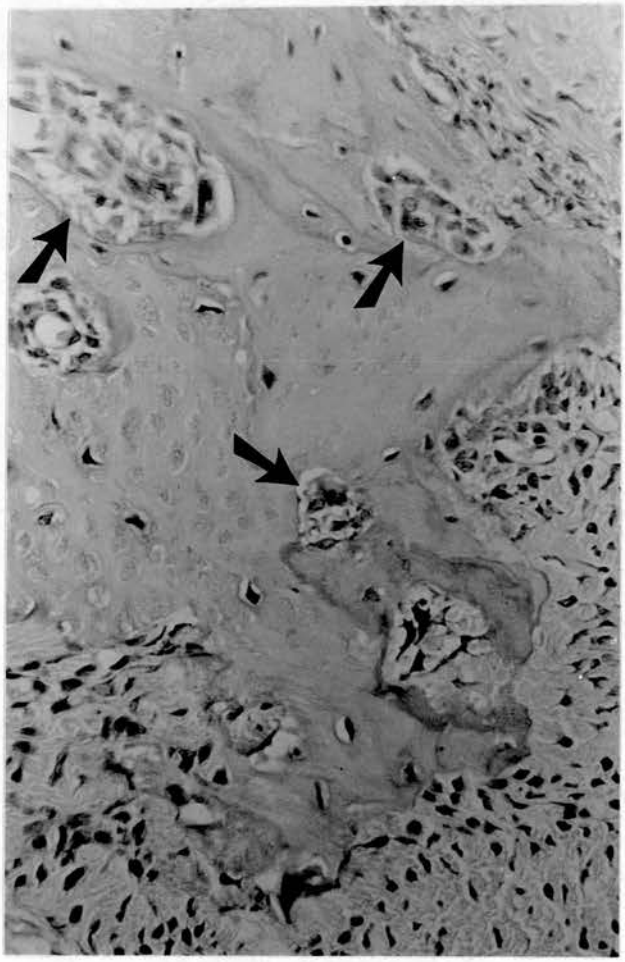


Fig 4.7 Section of crestal bone from the first mandibular molar region of a 10^7 cfu inoculated C57 mouse showing active bone resorption with osteoclasts (indicated by arrows). Magnification x300. Stained H & E.

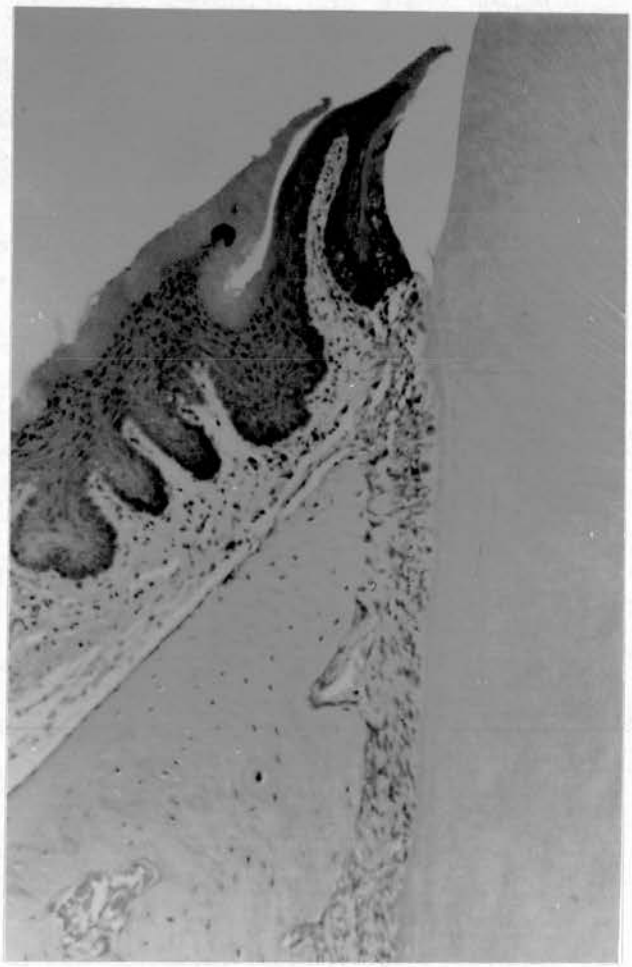


Fig 4.8 Buccolingual section through the lingual gingival sulcus in the lower left first molar region of a 6 week old uninoculated beige mouse showing the base of the epithelial attachment located at the ACJ.
Magnification x120. Stained Azure.

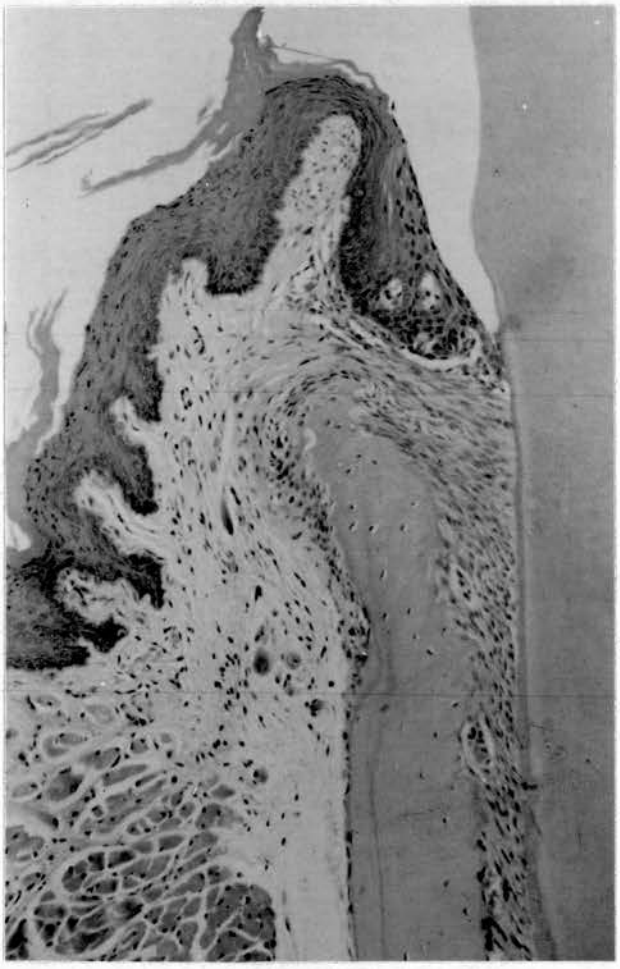


Fig 4.9 Buccolingual section through the buccal gingival sulcus in the lower left first molar region of a 36 week old uninoculated beige mouse showing the epithelial attachment partially located on cementum.
Magnification x120. Stained H & E.

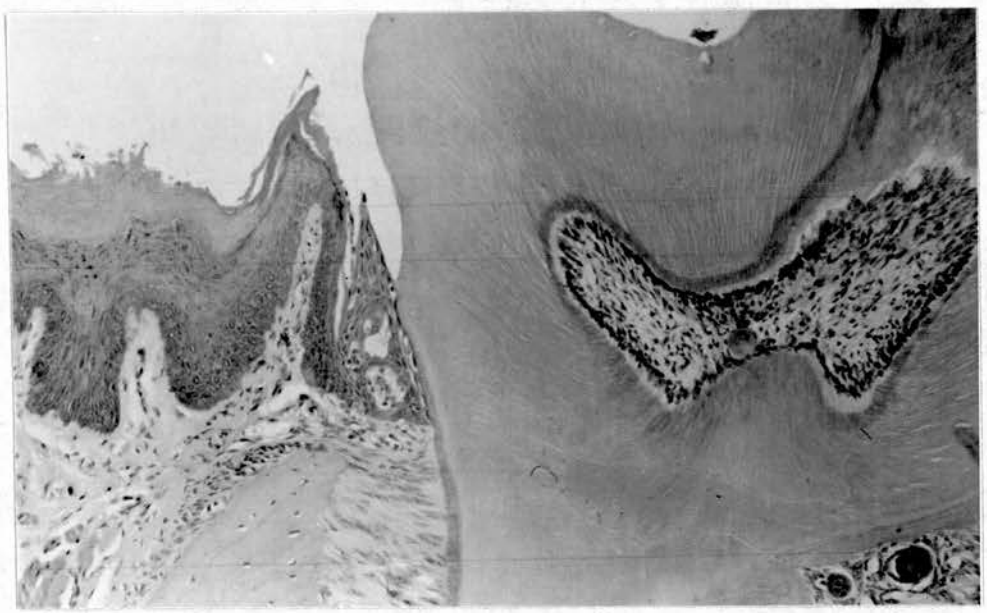


Fig 4.10 Buccolingual section through the lingual gingival sulcus in the lower left first molar region of a 52 week old uninoculated beige mouse showing the epithelial attachment largly located on cementum.
Magnification x120. Stained H & E.

4.4 D I S C U S S I O N

It was surprising that no consistent histological pattern emerged which could be related to the bone loss findings of either the main study or the immunisation experiment, though a more detailed quantitative assessment could have been more informative. The reasons for this are not clear but one possibility is that periodontal destruction is essentially cyclic in nature (Socransky et al, 1984), resulting in histological findings that were not comparable, even in age, strain and treatment matched mice.

The results for beige mice at different ages were consistent with the findings of Gilmore and Glickman (1959) in that both the degree of inflammation and loss of epithelial attachment increased with time. However, the severity of both these features was less than shown in the 1959 publication, possibility due to the difference of genotype.

In conclusion, the histological findings, rather than helping to explain the bone loss results, have posed new questions, in particular with regard to the value of histological parameters in assessing experimental periodontal disease in the mouse. The investigators who first developed the A. viscosus mouse model (Fitzgerald et al, 1981; Fitzgerald & Birdsell, 1982) have not published any histological findings so no comparison with the current work is possible.

CHAPTER 5

RECOVERY OF A. VISCOSUS

5.1 INTRODUCTION

Fitzgerald et al (1981) demonstrated that previously inoculated A. viscosus was recoverable only from the teeth and that, while there was a 'dramatic rise' in the number of organisms recovered over the first four weeks after oral inoculation, a plateau was reached after six weeks. These authors also found that the level of colonisation was 'similar' regardless of the initial inoculum and were unable to recover A. viscosus from uninoculated animals. In the present study, the level of colonisation of the teeth by A. viscosus was assessed at the end of the experimental period only, 12 weeks after oral inoculation.

5.2 MATERIALS AND METHODS

Recovery of A. viscosus was performed as described in Section 2.3, on two mice of each genotype (with the exception of +/xid) and each inoculum group.

5.3 RESULTS

A. viscosus was never recovered from uninoculated mice and always recovered from inoculated mice.

Analysis of variance for recovery count showed that variation between quadrants and between mice was not significantly greater than that between repeat measurements within quadrants for each genotype/inoculum group. Subsequent analysis was therefore restricted to differences between genotypes and inoculum groups only. This analysis is summarised in Table 5.1. Statistically significant differences between inoculum groups were found, but

only for BALB/c, C57 and ob/ob. No consistent pattern emerged and, furthermore, these differences were trivial compared to the thousand-fold range in number of organisms inoculated. On the other hand, statistically significant differences between genotypes were found for all inoculated groups, with BALB/c showing consistently low counts that were always significantly lower than +/bg, bg/bg and CBA. The significance of comparisons between genotypes over all inoculum groups is summarised in Table 5.2. Spearman rank correlation coefficients over the seven genotypes between mean recovery count (over all inoculated groups) and bone loss in uninoculated controls and in mice inoculated with 10^6, 10^7 and 10^9 cfu were all negative but statistically non-significant (-0.32, P = 0.48; -0.26, P = 0.62; -0.43, P = 0.34; -0.14, P = 0.76 respectively using two-tailed probabilities).

Table 5.1 Mean A. viscosus recovery counts in cfu x 10^3 per quadrant for the different genotype/inoculum groups, and the results of one-way analysis of variance between inoculum groups within genotypes and between genotypes within inoculum groups.
n = number of mice x quadrant x triplicate (2 x 4 x 3) counts.

Genotype		10^6	10^7	10^9	F	Significance
BALB/c	n	24	24	24	6.5	0.003
	Mean	153.2	144.6	155.5		
	s.e.	2.9	1.8	1.9		
C57	n	24	24	24	12.8	0.000
	Mean	160.7	163.2	151.8		
	s.e.	1.5	1.9	1.6		
+/bg	n	24	24	24	0.4	0.645
	Mean	165.8	168.0	168.2		
	s.e.	2.5	1.3	2.0		
bg/bg	n	24	24	24	2.9	0.060
	Mean	163.8	162.0	169.2		
	s.e.	2.5	2.1	1.9		
ob/ob	n		24	24	8.2	0.006
	Mean		166.9	157.2		
	s.e.		2.5	2.3		
CBA	n	24	24	24	1.8	0.171
	Mean	165.5	158.0	162.0		
	s.e.	3.0	2.7	2.7		
xid/Y	n	24	24	24	1.5	0.233
	Mean	155.3	159.0	161.6		
	s.e.	2.4	2.7	2.6		
	F	4.6	12.6	8.8		
	Significance	0.001	0.000	0.000		

Table 5.2 Summary of statistically significant differences for *A. viscosus* recovery between genotypes (for all inoculated groups). Means are number of cfu x 10^3. * = $P < 0.05$.

Genotype:		BALB/c	C57	xid/Y	CBA	ob/ob	bg/bg	+/bg
Mean	Genotype							
151.1	BALB/c							
158.5	C57	*						
158.6	xid/Y	*						
161.8	CBA	*						
162.0	ob/ob	*						
165.0	bg/bg	*	*	*				
167.3	+/bg	*	*	*	*	*		

5.4 DISCUSSION

Inability to recover A. viscosus from uninoculated animals confirmed that this organism was not a normal oral commensal in the mice examined.

The differences in colonisation level between genotypes over all inoculum groups (Table 5.2) show that the mutant genotypes tended to have higher recovery counts than the three inbred strains. There is therefore some indication that immunological mutants may tend to support higher levels of colonisation of the teeth by A. viscosus than non-mutant genotypes. However, although counts for both +/bg and bg/bg were significantly higher than for C57 controls, ob/ob and xid/Y did not show significantly higher counts than their respective C57 and CBA controls. Whether such differences are mediated through immunological mechanisms or simply through, for example, differences in tooth surface area or habits such as hair chewing is, at present, a matter for speculation. In terms of disease experience, the lack of significant correlation between bone loss and A. viscosus recovery count over the seven genotypes indicates that differences between genotypes for bone loss were unlikely to have had their basis in differences of colonisation level.

The 10 and 1000-fold differences in initial inoculum size were not reflected in the final level of colonisation by A. viscosus. It can therefore be concluded that, over the range of inoculum size used, colonisation of the teeth ultimately

stabilised at essentially the same level for each genotype. The final level of colonisation was therefore not related to bone loss. Consequently, it seems likely that the consistently high bone loss associated with inoculations of 10^7 cfu (Figure 3.4) was the result of host response to the initial inoculum. This hypothesis is discussed in subsequent chapters.

One further point of interest is that while the final absolute numbers of A. viscosus recovered did not show consistent differences between inoculum groups, the actual area of tooth surface available for colonisation was greatest in 10^7 inoculated animals due to the high level of bone loss. Assuming the same efficiency of recovery for all inoculum groups, the number of organisms per unit area of available tooth surface in this inoculum group must therefore have been less than in the 10^6 and 10^9 inoculated groups. There is no obvious explanation for this, but it is possible that some of the host defence and/or destructive processes in the 10^7 group, such as extracellular enzyme release from gingival fluid polymorphs, antibodies in saliva and gingival fluid or lymphokine production, could be responsible.

It is thus apparent that results presented here for the seven genotypes are comparable with the findings of Fitzgerald et al (1981) for BALB/c. The present study shows, in addition, that a more or less consistent pattern of A. viscosus recovery may be associated with different genotypes.

CHAPTER 6

POLYMORPHS

6.1 I N T R O D U C T I O N

Polymorphonuclear leucocytes (polymorphs) are found in the gingival tissues and saliva of both healthy and diseased mouths. However, as in other infections having an acute inflammatory component, the host response to subgingival plaque microorganisms is characterised in part by a marked increase in the number of these cells. Among them, numerically the most important are the neutrophil granulocytes (neutrophils) which constitute approximately 98% of polymorphs and approximately 70% of all blood leucocytes.

Polymorph cytoplasm contains two types of lysosomal granule. First, there are the azurophilic or primary granules, so-called because of their blue appearance when stained with Wright's stain and because they are the first granules to appear during neutrophil maturation. They contain lysozyme, acid hydrolases, neutral proteases such as elastase, cationic proteins, myeloperoxidase, acid mucopolysaccharide and beta-glucuronidase. Second, there are the specific or secondary granules, so-called because they are found only in neutrophils and appear after primary granules. They contain lysozyme, lactoferrin, B12 binding protein and neutral proteases such as collagenase. In the mature neutrophil, the specific granules are the most numerous (Murphy, 1976a)

It is generally accepted that extracellular lysosomal enzyme release is associated with tissue damage. Two possible mechanisms of such 'degranulation' are phagocytic release and secretion (Baggiolini et al, 1978), both of which depend on fusion of

lysosomes with the plasma membrane. Phagocytic release is triggered by the ingestion of particulate matter and, the larger the particle, the greater the number of lysosomal granules released (Miller et al, 1984). Convincing evidence of a true secretory mechanism for specific granules is still lacking but, as pointed out by Miller et al (1984), it is tempting to hypothesise that it does exist since lysozyme, lactoferrin and collagenase are often found extracellularly. Release of lysosomal granules may be triggered *in vitro* by complement-derived chemotactic factors released by bacteria (Murphy, 1976b). Lysosomal enzymes are also released following polymorph death (Murphy, 1976b).

6.1.1 ORAL POLYMORPHS

It has long been known that neutrophils are present in human saliva and it has been reasonable to assume that these are ultimately derived from blood leucocytes. Nevertheless, early work was unable to demonstrate a correlation between salivary and blood leucocyte counts in healthy subjects and those with both haematological and non-haematological malignancies (Stephens & Jones, 1934). Calonius (1958), however, compared salivary leucocyte counts from patients with both normal and inflamed gingiva, carious teeth and edentulous mouths. Counts were highest in subjects with gingival inflammation and lowest in edentulous cases, suggesting that salivary leucocyte count might be useful in assessing gingival pathology. Confirmation of the relationship between tooth presence and salivary leucoctye count was provided by Lantzman & Michman (1970) who compared counts before and after tooth extraction. They found that a drop in salivary leucocyte

numbers, sometimes to zero, occurred following extraction, suggesting that the gingival crevice is the main source of these cells.

Löe (1961) sealed the orifice of the healthy gingival crevice in dogs and demonstrated a build up of neutrophils and epithelial cells within the crevice, indicating that epithelial desquamation and neutrophil migration into the crevice are continuous processes in the healthy state. An ultrastructural study of clinically normal crevicular tissues in beagle dogs has demonstrated neutrophils not only in the lamina propria but also migrating through the basal lamina and within the epithelium itself (Garant & Mulvihill, 1971a). In germ free rats, neutrophils have been seen routinely within the intercellular spaces of the crevicular epithelium at all levels of attachment (Yamasaki et al, 1979). Thus, the passage of neutrophils into the gingival crevice cannot be attributed to the presence of microbial plaque alone, implying that, at least under certain circumstances, their presence is physiological rather than pathological. It has been suggested that the possibility of chemotactic attraction to food antigens should not be ignored (Taubman et al, 1981). Nevertheless, recruitment of neutrophils to the crevice is generally believed to be largely mediated either by bacterially derived chemotactic factors or through activation of the complement system (Tempel et al, 1970; Lindhe & Hellden, 1972; Mary et al, 1972; Hellden & Lindhe, 1973; Kraal & Loesche, 1974; Taichman & Baehni, 1977).

The relationship between neutrophils and gingival pathology has been the subject of a number of investigations. Payne et al

(1975), in studies on the early stages of experimental gingivitis in man, demonstrated a statistically significant increase in the numbers of neutrophils in the junctional epithelium after 2-4 days compared with clinically normal gingiva. Theilade et al (1966) found that the number of neutrophils increased with the development of gingivitis but rapidly decreased again with the reinstitution of oral hygiene measures. Work by Garant & Mulvihill (1971b) demonstrated the presence of many neutrophils within dilated intercellular spaces of chronically inflamed crevicular epithelium of beagles. Many neutrophils were disrupted with apparent extrusion of lysosomal granules and the crevicular epithelium displayed decreased numbers of intercellular contacts. These findings suggested that increased neutrophil emigration leads to increased antigen exposure to the underlying connective tissue.

The Orogranuloctye Migratory Rate (OMR), as measured by counting the number of leucoctyes in 12 sequential 30-second oral saline rinses (Klinkhamer & Zimmerman, 1969), has been proposed as a useful indicator of gingivitis. While it is true that patients with gingival inflammation do tend to have higher OMRs than those with healthy gingivae (Woolweaver et al, 1972), the value of OMR as an index of severity of periodontal disease in an individual patient seems limited (Hase & Reade, 1979). Golub et al (1981) and Singh et al (1982) used an *in vitro* chemotaxis assay for crevicular polymorphs which employed casein as a chemoattractant. They found the influx of polymorphs in patients with both gingivitis and chronic periodontitis to be greater than that of

polymorphs in controls. It is hoped that this assay may prove to be an important diagnostic aid for studying polymorph migration in both periodontal and systemic diseases.

6.1.2 PROTECTIVE ROLE

According to Miller et al (1984) the increase in number of polymorphs with increasing gingival inflammation can be viewed as a host protective response against bacterial plaque. In studies of mono-infected rats (Garant, 1976) a polymorph wall was seen to form between plaque and host tissue and, while plaque bacterial phagocytosis was not a prominent feature, the wall was thought to prevent bacterial products from entering the tissues. Electron microscopic studies of neutrophils and plaque microorganisms by Baehni et al (1977), Taichman et al (1977) and Tsai et al (1978) demonstrated that not only were the polymorphs in close association with the plaque mass, but that pseudopodal extensions from the polymorphs penetrated the mass. The polymorphs contacting the plaque showed a decrease in number of lysosomal granules and phagocytosis was noted when the cells contacted individual organisms.

More direct evidence for a protective role for polymorphs comes from studies of disorders of polymorph function. Attstrom & Schroeder (1979) demonstrated increased apical extension of subgingival plaque in neutropenic dogs and suggested that polymorphs may prevent the progress of subgingival plaque formation. Human sufferers of Chediak-Higashi syndrome (CHS) have an increased susceptibility to infection which is associated with granulocytopenia (Blume et al, 1968), an abnormality of

granuloctye chemotaxis (Clark & Kimball, 1971), a delay in the killing of phagocytosed bacteria, particularly staphylococci (Root et al, 1972), and an abnormal distribution of enzymes in leucocyte lysosomes (Wolff et al, 1972). In those cases with oral status evaluation, severe gingivitis, caries and varying degrees of oral ulceration have been noted (Tempel et al, 1972; Hamilton & Giasanti, 1974) and, of ten cases for whom intraoral radiographs had been taken, seven showed severe early periodontal destruction resulting in premature tooth loss.

Beige mice were found to have increased susceptibility to infection caused by Staphylococcus aureus, Streptococcus pneumoniae and Candida albicans (Elin et al, 1974). Specific studies to further characterise beige mouse leucocyte function were undertaken by Gallin et al in 1974. Using peritoneal exudate cells from black control (NIH stock C57B6/6N) and beige (NIH stock C57Bl/6/6JBg) mice, these authors found that the beige leucocytes showed significantly reduced chemotaxis and decreased bactericidal capacity against Staphylococcus aureus and some 'group D streptococci', though this latter finding was not statistically significant after 90 minutes. The killing of Escherichia coli by beige leucocytes "appeared to be less effective than that of normal cells" but the phagocytic uptake of these organisms by cells of both genotypes was not significantly different, leading these workers to conclude that the abnormality in CHS leucocytes was related to a defect in early intracellular killing. It is interesting, however, that granulocytopenia, a characteristic findings in CHS in man (Blume et al, 1968), is not a feature of

beige mice (Bennett et al, 1969).

Other conditions where impaired neutrophil function and increased periodontal disease occur together are chronic granulomatous disease (Simchowitz et al, 1980), localised juvenile periodontitis (Cianciola et al, 1977; Clark et al, 1977; Lavine et al, 1979; Vandesteen et al, 1981; Van Dyke et al, 1981) and other aggressive early onset periodontal diseases (Page et al, 1983a & b). Patients with Job's syndrome were shown to have depressed polymorph chemotaxis (Hill et al, 1974), and Van Dyke et al (1985) observed that such patients exhibited severe gingivitis and alveolar bone loss. Bissada et al (1982) and Cianciola et al (1982) have demonstrated that, among juvenile diabetes mellitus sufferers, a subpopulation exhibiting reduced neutrophil chemotaxis exists. Also, polymorphs from diabetics with severe periodontal disease have been shown to have a reduced chemotactic response relative to diabetics with mild periodontal disease or non-diabetics with periodontal disease (Manouchehr-Pour et al, 1981). Polymorphs from patients with inflammatory bowel disease appear to have altered glucose metabolism, possibly through interaction with circulating immune complexes. When faced with severe gingival challenge (eg necrotising ulcerative gingivitis), these patients may have difficulty in containing the local infection resulting in rapid loss of alveolar bone (Lamster et al, 1978; 1982).

These findings all seem to point to defective polymorph function as being associated with increased disease experience, thus suggesting that the neutrophil is essentially protective.

6.1.3 DESTRUCTIVE ROLE

As summarised by Miller et al (1984) "it is the extracellular release of lysosomal granules by the recruited polymorphs that has been suggested to be in part responsible for tissue destruction observed in periodontal disease".

Jensen et al (1966) showed that the application of oral Veillonella endotoxin to human abraded skin windows resulted in immediate migration of neutrophils to the treated site and peak phagocytic activity in 4-6 hours. It is possible for endotoxin to produce similar effects in the gingiva, perhaps via complement activation. Endotoxin has been shown to pass through intact crevicular epithelium (Schwartz et al, 1972) and its presence in gingival exudate has been found to correlate with the degree of clinically and histologically detectable inflammation (Simon et al, 1970; 1971). Taichman et al (1966) reported that intradermal injection of normal rabbits with human plaque resulted in localised abscesses and histological signs of neutrophil mediated phagocytosis of bacteria, degranulation, increased vascular permeability and haemorrhage. On the other hand, a similar injection into leucopenic animals resulted in no local signs but, after 24 hours, signs of septicaemia were noted. This implies that leucocytes, while being essential for local defence, can cause tissue damage by local enzyme release.

In support of this concept, various oral bacteria have been shown to cause neutrophil degranulation (Baboola & Powell, 1972; McArthur et al, 1976; Taichman & McArthur, 1976; Baehni et al, 1977; Taichman et al, 1977; Tsai et al, 1978). Pooled human supra- and subgingival plaque incubated with human polymorphs

showed a dose dependent relationship with lysosomal enzyme release (Taichman et al, 1977). The addition of fresh autologous serum augmented enzyme release in response to both supra- and subgingival plaque but was not an absolute requirement. Indeed, Passo et al (1980) demonstrated that A. viscosus could be killed by polymorphs without serum. This serum enhancement however implies a modulating effect by complement components.

Immune complexes associated with A. viscosus and Streptococcus mutans have been found to trigger polymorph degranulation (McArthur et al, 1976). Rizzo & Mitchell (1966) and Ranney & Zander (1970) showed that experimental gingivitis and periodontal inflammation could be induced by local formation and deposition of immune complexes in animal studies, and complement components and antibody against plaque antigens have been found in diseased human tissues (Berglund, 1971; Taubman, 1974). Clagett & Page (1978), however, were unable to find significant amounts of immune complex in periodontally diseased dogs or humans using immunofluorescent techniques. Thus the role of the immune complex is as yet unclear.

Free lysosomes have been detected in the intercellular spaces of chronically inflamed human gingiva (Freedman et al, 1968). Thilander (1963) found that application of a leucocyte homogenate to healthy human marginal gingiva resulted in widening of the intercellular spaces of pocket epithelium in much the same way as caused by bacterial hyaluronidase. Such widening may facilitate passage of destructive substances into the connective tissue.

Ohlsson et al (1974) demonstrated free extracellular

polymorph collagenases and elastase in the crevices of inflamed human gingiva and, while they were detected at healthy sites, they were found at only 15% of the concentration at diseased sites, most of the collagenase and elastase at healthy sites being bound to serum protease inhibitors. Another study (Kowashi et al, 1979) showed a significant increase in free collagenase and neutral protease concentrations in gingival washings from inflamed human gingival crevices after 21 days of no tooth brushing, with a concomitant increase in numbers of polymorphs. While it is true that polymorph collagenase is immunologically similar to epithelial and connective tissue collagenase, the presence of collagenase in periodontal disease, along with inflammatory cell infiltrate and collagen loss, suggests that it is primarily of neutrophil origin (Miller et al, 1984).

Schroeder & Lindhe (1980) investigated the pathology of destructive experimental periodontitis in dogs and found that rapid alveolar bone destruction was the consequence of intense acute inflammation and junctional epithelial ulceration. A chronic inflammatory infiltrate was noted with low osteoclast density at the bone surface. This experiment thus implies that the role of polymorphs in alveolar bone loss is an active one.

6.1.4 POLYMORPH FUNCTION

The functions of polymorphs are related to their ability to engulf and destroy microorganisms, ingestion of foreign particles being associated with a marked increase in the uptake of oxygen (Sbarra & Karnovsky, 1959). This is referred to as the metabolic or respiratory burst because normally the cell is relatively

dormant metabolically. It is generally accepted that the primary oxidase responsible for the respiratory burst is located on the outer surface of the polymorph plasma membrane (Briggs et al, 1975; Goldstein et al, 1977; Dewald et al, 1979). Initiation of the respiratory burst occurs on membrane stimulation prior to ingestion and degranulation (Rossi et al, 1972). Studies by Korchak and Weissmann (1978) have provided evidence that alteration of polymorph membrane potential may be the triggering stimulus. Activation of the hexose-monophosphate shunt, the other major component of the respiratory burst aside from the primary oxidase of phagocytosis, has been thought to be related to nicotinamide adenine dinucleotide phosphate dehydrogenase (NADPH) oxidase and NADPH-linked lactic dehydrogenase (Evans & Karnovsky, 1962), as well as the glutathione cycle (Karnovsky et al, 1971). The details of the possible complex interrelationships of these pathways are summarised in Klebanoff & Clark (1978).

The products of the respiratory burst include superoxide anions (O_2^-), hydrogen peroxide (H_2O_2), singlet oxygen ($^1O_2$) and hydroxyl radicals (OH^-). These toxic oxygen derivatives not only kill microorganisms but also attack tumour cells and parasites (Klebanoff & Clark, 1978). In association with the oxygen burst, following bacterial ingestion, degranulation occurs. The studies of Cohn & Hirsch (1960) demonstrated that lysosomal enzymes sequestered in lysosomes are then discharged from granules into phagocytic vacuoles or secondary lysosomes.

The systems within polymorphs that are responsible for antimicrobial action are either oxygen dependent, such as the myeloperoxidase system, or oxygen independent, such as lysozyme, lactoferrin and neutral proteases. The myeloperoxidase (MPO) system requires halide and hydrogen peroxide as well as the enzyme myeloperoxidase. This enzyme catalyses the reaction of hydrogen peroxide and chloride to generate the potent oxidant hypochlorous acid which is powerfully microbicidal agent. The MPO system has been found to be less effective when any of the three components are missing (Rosen & Klebanoff, 1979). In addition, enzymes that may be produced by ingested bacteria, such as catalase, can limit the capacity of the system by decreasing available hydrogen peroxide (Murphy, 1976b). The four by-products of the respiratory burst (hydrogen peroxide, superoxide anion, hydroxyl radicals and singlet oxygen) appear to have their own antimicrobial activity augmented by myeloperoxidase and halide (Murphy, 1976b). MPO binds to target microorganisms and this has been shown to be important for myeloperoxidase-mediated killing of both bacteria (Selvaraj et al, 1978) and fungi (Wright et al, 1983) in vitro. It is thought that increased microbicidal activity of the target-bound enzyme may result from the generation of hypochlorous acid in close proximity to the microbial substrates (Wright & Nelson, 1985).

A sensitive way to measure the respiratory burst is based on the finding that stimulated phagocytes generate photons of light which can be detected as chemical luminescence or chemi-luminescence (CL) (Fromtling et al, 1981; De Chatelet et al,

1982). This light emission has been shown to depend both on superoxide and myeloperoxidase-catalysed reactions and is a measure of phagocytic as well as metabolic activity in the stimulated cells (Trush et al, 1978). There are two main light amplifiers in common use in CL studies, luminol (5-amino-2, 3-dihydro-1, 4 pthalazinedione) and lucigenin (10,10-dimethyl-bis-9, 9-biacridinium nitrate). Muller-Peddinghaus (1984) found that lucigenin appeared to react mainly with the superoxide anion, the first of the generated reactive oxygen species, while luminol appeared to react mainly with hydrogen peroxide and the singlet oxygen. This author suggested that lucigenin records cell activity via the respiratory burst more reliably than does luminol which depends on the generation of the chain reaction intermediate H_2O_2 and on its three main catalysts, catalase, MPO and glutathione. However, many laboratories use both these amplifiers. In vitro stimulation of polymorph CL can be achieved by fungi, bacteria, particulate antigen such as zymosan or soluble agents such as PMA (phorbol-12-myristate-13-acetate).

6.1.5 SCOPE OF PRESENT STUDY

Polymorphs from mice of various genotypes used in the present study were tested to see whether polymorph function could be related to the level of naturally occurring and induced disease. The tests used were an MPO assay and CL using zymosan and PMA as stimulators and both luminol and lucigenin as light enhancers.

6.2 MATERIALS AND METHODS

6.2.1 POLYMORPHS

Polymorphs were harvested from peritoneal exudate of mice of the following genotypes, BALB/c, C57, +/bg, bg/bg, ob/ob and CBA. All mice were 7 week old uninoculated males. Peritoneal exudate cells were obtained according to the method of Gallin et al (1974). Mice were given an intraperitoneal (IP) injection of 4 ml sterile 10% sodium caseinate (Koch-Light Ltd, Colnbrook, Berks, UK) in isotonic saline and, immediately prior to harvesting the peritoneal exudates 16 hours later, all the animals were given a 2 ml IP injection of 20 μ ml^{-1} heparinised saline (heparin from Sigma Chemical Co Ltd, Poole, UK). The animals were then sacrificed by cervical dislocation, the peritoneal cavity opened and the exudate aspirated.

The exudates were spun at 2500 g at 4°C for 5 minutes and the pellet resuspended in 10 ml cold sterile phosphate buffered saline (PBS) (Flow Labs, Rickmansworth, UK). 100 μl of each cell suspension was placed in 300 ul of cell counting fluid and a count was made for each in a Neubauer Counting Chamber (Weber Ltd, England). The cell concentrations were then adjusted to 5×10^6 ml^{-1} for both MPO and CL assays. Differential counts were made by placing 50 μl of a 5×10^6 cells ml^{-1} cell suspension and 300 μl cold PBS in a cytospin (Shandon Southern Ltd, Runcorn, UK). The resultant slide was stained with Leishman's stain, viewed via a dissecting microscope (Zeiss Ltd, Wellwyn Garden City, UK), and the numbers of different cell types present were counted. Viability testing was carried out using trypan blue exclusion, by

mixing equal volumes of cell suspension and 0.5% trypan blue in 0.9% saline. After 5 minutes the numbers of stained and unstained cells were counted. The unstained cells were considered viable.

6.2.2 MYELOPEROXIDASE ASSAY

This test was performed on three separate occasions, each time using cells from one mouse of each of the six genotypes. On each occasion 200 µl of the fresh 5 x 10^6 ml^{-1} cell suspension was spun at 10,000 g and resuspended in 200 µl acetate buffer (see Appendix). This was stored at -20°C till the next day when the cells were thawed and then frozen and thawed 3 times followed by centrifugation at 10,000 g for 25 minutes. The supernatant was decanted and used for the assay. To 1 ml of acetate buffer was then added 0.05487g of 2,2'-azino-di (3 ethylbenzthiazoline sulphonic acid) (ABTS) and this was diluted 1:97 in acetate buffer. All test samples were run with a blank with the appropriate cuvettes containing:

	Test	Blank
Acetate buffer/ABTS	0.97 ml	0.97 ml
H_2O_2	0.01 ml	0.01 ml
Acetate buffer	-	0.02 ml
Cell supernatant	0.02 ml	-
	1.00 ml	1.00 ml

First, the H_2O_2 and cell supernatant/acetate buffer were placed on opposite sides of the cuvettes. Only then was 0.97 ml of the acetate buffer/ABTS added vigorously to each cuvette and the optical density (OD) measured at 412 nm for 3-5 minutes in a spectrophotometer (Pye Unicam, Cambridge, UK, model SP8200). During this experiment the myeloperoxidase acted on the H_2O_2

in the acid conditions and its activity was recorded by a colour change in the ABTS. MPO activity was calculated by the following equation:

$$\frac{OD}{32.4} \times \frac{\text{storage volume}}{\text{assay volume}} \times \frac{1}{\text{no of cells stored (in millions)}}$$

with units = units/10^6 cells/minutes. (Klebanoff, 1965).

6.2.3 CHEMILUMINESCENCE ASSAYS

These tests were performed in duplicate on three separate occasions. On each occasion cells from one mouse of each of six genotypes were tested. The stimulants used were prepared as follows.

(a) Zymosan (Sigma Chemical Company, Poole, UK): this was incubated for 30 minutes at 37°C in 10% autologous or AB serum and spun and washed once. The zymosan was then resuspended in Hank's Balanced Salt Solution (HBSS) (Flowlabs, Rickmansworth, UK) to a concentration of 20 mg ml^{-1} and stored frozen (-40°C) till required. The working concentration of 10 mg ml^{-1} was achieved by the addition of HBSS.

(b) PMA (Sigma Chemical Company, Poole, Dorset): this was prepared by dissolving 5 mg PMA in 2.5 ml dimethyl sulphoxide (Sigma Chemical Company, Poole, UK) (DMSO) to give a 2 mg ml^{-1} stock solution which was divided into 10 µl aliquots and then stored frozen (-40°C). When required, aliquots were thawed and HBSS added to give the required working concentration of 20 µg ml^{-1}.

The enhancers were prepared as follows:-

(a) Luminol. Stock at a concentration of 10^{-3} M was prepared by adding 0.0177 g luminol (Sigma Chemical Company, Poole, UK) to 1 ml DMSO and this was then diluted to 100 ml in HBSS. The stock solution was diluted in HBSS to the working concentration of 4×10^{-5} M.

(b) Lucigenin. This was prepared by adding 0.05105 g lucigenin (Sigma Chemical Company, Poole, UK) to 1 ml HBSS and then adding a further 100 ml of HBSS to produce a stock solution at a concentration 10^{-3} M which was stored at -20°C. This was also the working concentration.

To perform the assay, stimulant, HBSS and enhancer were added to the assay tubes not more than 30 minutes before the test was read. The cells were added at the last moment before reading began. The tubes contained the following:-

Reagent	Volume	Concentration when added	Final Concentration
HBSS	100 µl		
Cells	200 µl	5×10^{6} ml^{-1}	1×10^{6} ml^{-1}
Luminol	200 µl	4×10^{-5} M	8×10^{-6} M
or Lucigenin	200 µl	15×10^{-4}M	3×10^{-4} M
Zymosan	500 µl	10 mg ml^{-1}	5 µg ml^{-1}
or HBSS	400 µl		
+ PMA	100 µl	20 µg ml^{-1}	2 µg ml^{-1}

The light emitted was detected and measured using an LKB 1251 luminometer (LKB Instruments Ltd, South Croydon, UK).

6.3 R E S U L T S

6.3.1 POLYMORPH FUNCTION TESTS

The mean per cent polymorphs among peritoneal exudate cells and mean per cent viability of these cells are shown for each genotype in Table 6.1. Tables 6.2 and 6.3 respectively show mean MPO activity and chemiluminescence for each genotype. There was a consistently negative association between chemiluminescence and MPO activity over all genotypes, one correlation coefficient being significantly different from zero (Table 6.4). A nested analysis of variance (Table 6.5) for myeloperoxidase activity and chemiluminescence showed that there were statistically significant differences between genotypes for both tests. For chemiluminescence, there were also statistically significant differences between stimulants and between enhancers, but the variation between mice was not signficantly greater than that between duplicate measurements within mice. Reference to Table 6.3 shows that zymosan tended to induce higher chemiluminescence values than PMA, and that lucigencin was a much more potent enhancer than luminol.

Table 6.1 Mean per cent polymorphs among peritoneal exudate cells and per cent viability of peritoneal exudate cells (n = 3 mice per genotype).

Genotype	Mean % Polymorphs ± se	Mean % Viability ± se
BALB/c	74 ± 2.5	93 ± 2.6
C57	78 ± 2.4	96 ± 2.0
+/bg	75 ± 2.5	95 ± 2.2
bg/bg	76 ± 2.5	96 ± 2.0
ob/ob	75 ± 2.5	97 ± 1.7
CBA	74 ± 2.5	98 ± 1.4

Table 6.2 Mean myeloperoxidase activity in units as defined in Section 6.2.2.
(n = 3 mice per genotype).

Genotype	Mean MPO ± se ($x10^3$)
BALB/c	5.97 ± 0.27
C57	1.10 ± 0.11
+/bg	6.07 ± 0.37
bg/bg	13.27 ± 0.71
ob/ob	11.06 ± 0.05
CBA	7.41 ± 0.18

Table 6.3 Mean chemiluminescence ± se (m Volts)
t = time in seconds to peak
n = 6 (duplicate tests on 3 mice per genotype).

Genotype		Zymosan/Luminol	Zymosan/Lucigenin	PMA/Luminol	PMA/Lucigenin
BALB/c	Mean ± se	33.85 ± 0.40	141.50 ± 0.12	45.35 ± 0.08	61.95 ± 0.20
	t	512	1404	234	546
C57	Mean ± se	26.30 ± 0.08	75.95 ± 0.12	30.53 ± 0.22	43.37 ± 0.19
	t	512	1404	234	546
+/bg	Mean ± se	26.68 ± 0.12	115.85 ± 0.44	40.32 ± 0.07	46.38 ± 0.03
	t	512	1404	156	468
bg/bg	Mean ± se	4.88 ± 0.03	30.47 ± 0.07	0.56 ± 0.02	0.81 ± 0.01
	t	512	1560	156	546
ob/ob	Mean ± se	16.08 ± 0.56	68.77 ± 0.28	40.73 ± 0.20	33.32 ± 0.07
	t	512	1560	156	546
CBA	Mean ± se	19.57 ± 0.07	129.88 ± 0.16	47.48 ± 0.06	47.68 ± 0.10
	t	512	1560	156	546

Table 6.4 Spearman rank correlation coefficients (r_s) for the association between chemiluminescence and myeloperoxidase activity.
P values are two-tailed probabilities.

	Zymosan/Luminol	Zymosan/Lucigenin	PMA/Luminol	PMA/Lucigenin
r_s	-0.83	-0.60	-0.14	-0.60
P	0.04	0.21	0.79	0.21

Table 6.5 Nested analysis of variance for polymorph function tests. * indicates denominator for F ratios.

Test	Source of Variation	ss	DF	ms	F	Significance of F
Myeloperoxidase	Between genotypes	274.00	5	54.80	143.14	0.000
	Between mice within genotypes	4.59	12	0.38*	-	-
Chemiluminescence	Between genotypes	56605.67	5	11321.13	51597.58	0.000
	Between stimulants within genotypes	19154.77	6	3192.46	14550.07	0.000
	Between enhancers within stimulants	114408.94	12	9534.08	43452.83	0.000
	Between mice within enhancers	10.77	48	0.22	1.02	0.460
	Between duplicates within mice	15.80	72	0.22*	-	-

6.3.2 POLYMORPH FUNCTION AND BONE LOSS

Figure 6.1 illustrates two relationships between bone loss and MPO activity. Bone loss is expressed both as that found in uninoculated controls and as the ratio of that induced by 10^7 cfu to the control level. The Spearman rank correlation and its associated two-tailed probability are given for each relationship. Similarly, Figures 6.2 - 6.5 illustrate the association between bone loss and chemiluminescence. For Figures 6.1 - 6.5 the correlation of polymorph function with 10^7/control bone loss was consistently stronger than that with control bone loss. Correlation coefficients for MPO activity were negative while those for chemiluminescence were positive, three of the four associations between 10^7/control bone loss and chemiluminescence being statistically significant.

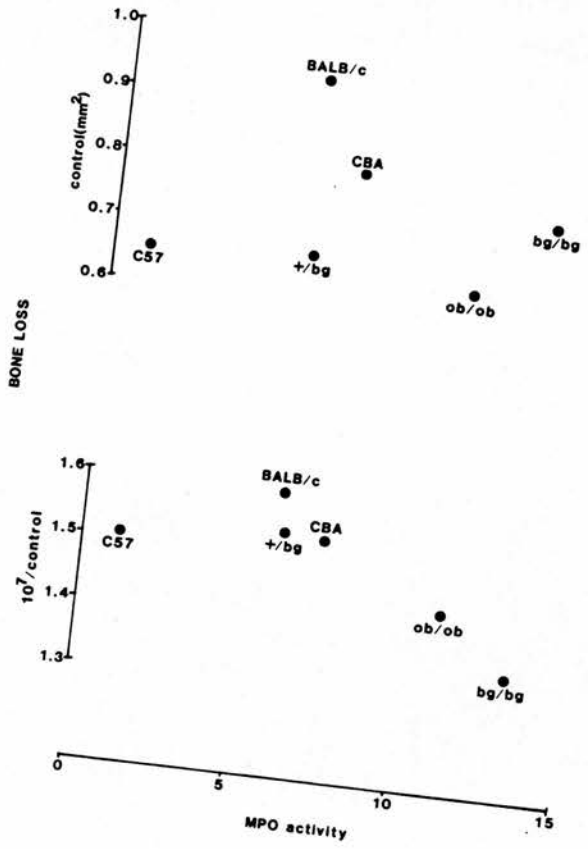


Fig 6.1 The relationship between bone loss and MPO activity (units/10^6 cells/minute x 10^{-3})

control bone loss: $r_s = -0.09$, $P = 0.87$
10^7/control bone loss: $r_s = -0.66$, $P = 0.16$

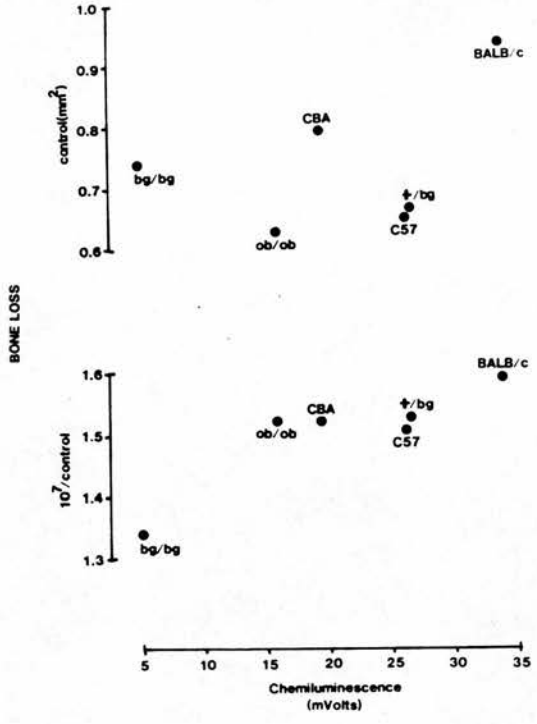


Fig 6.2 The relationship between bone loss and zymosan/luminal chemiluminescence.

control bone loss: $r_s = 0.37$, $P = 0.47$
10^7/control bone loss: $r_s = 0.66$, $P = 0.005$

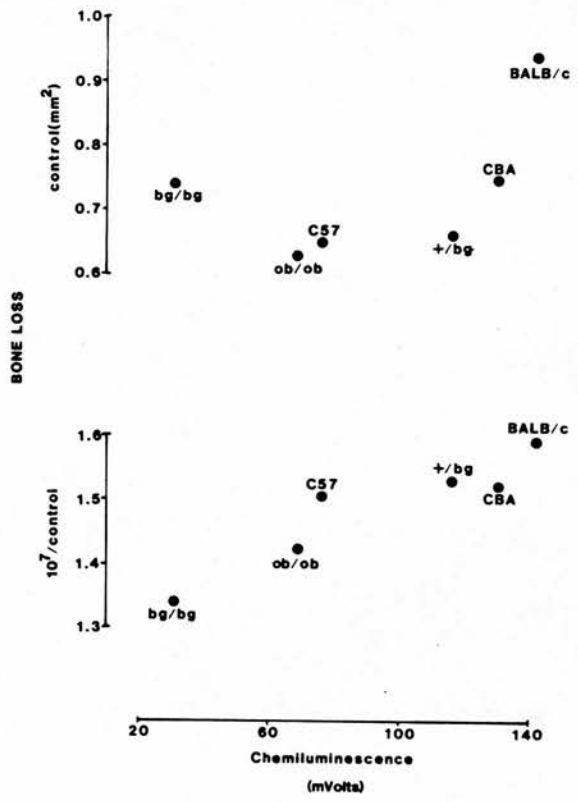


Fig 6.3 The relationship between bone loss and zymosan/ lucigenin chemiluminescence.

control bone loss: $r_s = 0.66$, $P = 0.16$
10^7/control bone loss: $r_s = 0.94$, $P = 0.005$

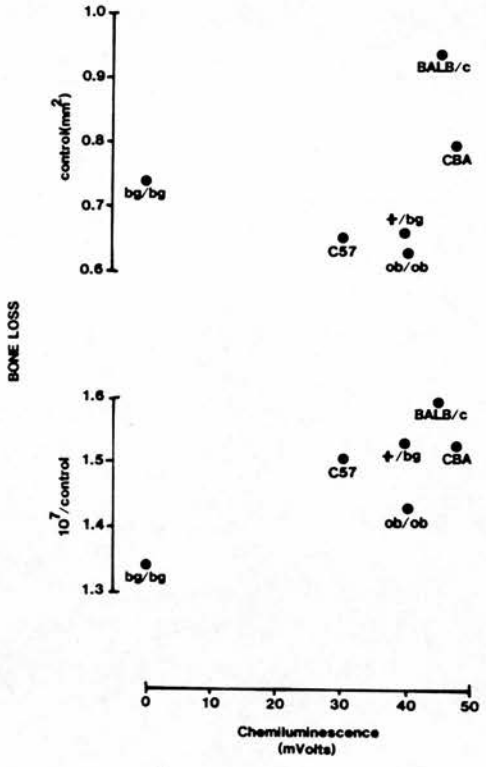


Fig 6.4 The relationship between bone loss and PMA/luminol chemiluminescence.

control bone loss: $r_s = 0.43$, $P = 0.40$
10^7/control bone loss: $r_s = 0.60$, $P = 0.21$

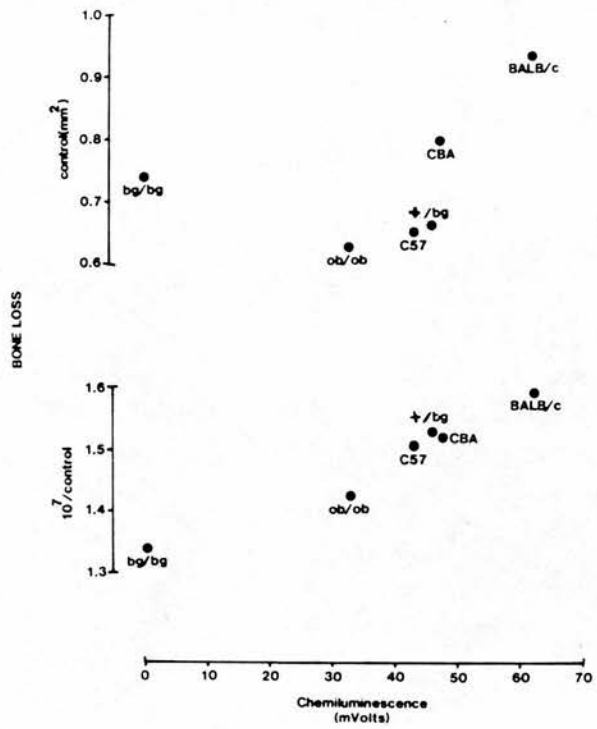


Fig 6.5 The relationship between bone loss and PMA/lucigenin chemiluminescence.

control bone loss: $r_s = 0.66$, $P = 0.16$
10^7/control bone loss: $r_s = 0.94$, $P = 0.005$

6.4 D I S C U S S I O N

All the tests described in this chapter were performed on uninoculated mice. Thus, the results show only the capacity of polymorphs from animals of the different genotypes to respond to standard stimuli and enhancers.

The use of peritoneal exudate polymorphs had the advantage that the cells were easily obtained and that the yield was high. However, there was a possible disadvantage in that the cells had already responded to a chemotactic stimulus before testing and may therefore have undergone some metabolic and functional changes.

The mean percentage of bg/bg polymorphs obtained (Table 6.1) did not differ significantly from that of the other genotypes. Thus, in view of the chemotaxis defects detailed by other authors in these mice (Gallin et al, 1974), any such defect must have affected all cell types. In addition, the cell condition was good as attested to by the high viability.

The standard errors for all the measurements made were small relative to their means indicating that sample size was adequate for statistical analysis. MPO activity was generally low (Table 6.2), consistent with the findings of Higgins et al (1978).

6.4.1 DIFFERENCES BETWEEN GENOTYPES

Both types of polymorph function test showed significant differences between genotypes. The increase in MPO acitivity from C57 through +/bg to bg/bg is consistent with a gene dosage effect for the mutant allele. It is known from previous work by Brandt et al (1975) and Kimball et al (1975) that lysosomes from beige

mice and CHS patients are very susceptible to mechanical breakage. MPO is an enzyme found in primary lysosomal granules and, even though secondary granules are more numerous in mature polymorphs (Murphy, 1976a), it is still logical that MPO levels may be higher in animals carrying the beige gene due to possible disruption as a result of washing and/or spinning procedures.

For chemiluminescence, on the other hand, +/bg showed either a similar or greater response than C57 but bg/bg by far the lowest response of all genotypes. It has been suggested in the past that the formation of large lysosomes in CHS patients and animal analogues may be related to altered membrane properties. Oliver et al (1976) reported cytochemical evidence for a possible lipid defect in beige cells and Chi et al (1975) found that the lamellar bodies of type II pneumocytes of beige mice had a three-fold increase in surface active phospholipid. As previously stated, it is generally accepted that the primary oxidase responsible for the respiratory burst in polymorphs is located on the outer surface of the polymorph plasma membrane (Briggs et al, 1975; Goldstein et al, 1977; Dewald et al, 1979). Chemiluminescence measures the respiratory burst, thus it may be that the presence of the homozygous beige gene results in some membrane change, possibly in amount or position of the primary oxidase, which may in turn affect the respiratory burst and thereby chemiluminescence.

No consistent relationship was found between the responses of ob/ob and C57 polymorphs in the different stimulant/enhancer groupings. There was, however, a tendency towards a negative association between chemiluminescence and MPO activity over all

genotypes (Table 6.4). As it was shown by Trush et al (1978) that light emission depends on both superoxide and myeloperoxidase catalysed reactions, the reasons for this negative association are therefore obscure.

Differences of polymorph function between mouse strains have also been described by Lamster et al (1979). These workers investigated the ability of polymorph supernatants to enhance the blastogenic response of mouse splenocytes to phytohaemagglutinin-P (PHA). BALB/c, A/J and C57BL/6J strains were tested. BALB/c and A/J mice had active supernatants capable of enhancing the blastogenic response to the mitogen, and splenocytes that responded to active supernatant in the presence of PHA. C57BL/6J mice, conversely, did not produce an active supernatant, and splenocytes from these animals failed to respond to active allogeneic supernatant in the presence of mitogen. These findings provide further evidence for genetic variation in polymorph function.

6.4.2 DIFFERENCES BETWEEN STIMULANTS AND ENHANCERS

The chemiluminescence produced by zymosan/luminol was less for all genotypes (except bg/bg) than that produced by PMA/luminol, while the chemiluminescence of zymosan/lucigenin was always greater than that of PMA/lucigenin. Differences between stimulants and enhancers therefore appear to exist.

The time taken to reach peak chemiluminescence (Table 6.3) shows that a peak was attained more rapidly with luminol than with lucigenin. This result is consistent with that of Muller-

Peddinghaus (1984). However, whereas that author found that luminol produced the greater absolute chemiluminescence, in the current work lucigenin produced the greater chemiluminescence in 11 of the 12 genotype/stimulant combinations. As pointed out by Muller-Peddinghaus, lucigenin reacts with the first of the generated reactive oxygen species, the superoxide anion radical (O_2^-). The shorter time to peak with luminol, which reacts with the chain reaction intermediate H_2O_2, is therefore to be expected. The cause, however, as to why the absolute value was almost always higher in lucigenin enhanced chemiluminescence is unknown.

6.4.3. ASSOCIATION BETWEEN POLYMORPH FUNCTION AND BONE LOSS

The most important finding is that a high MPO activity appears to be associated with protection from periodontal destruction induced by A. viscosus inoculation, whereas high chemiluminescence activity appears to be associated with high levels of destruction (see Figures 6.1-6.5). The biological significance of these findings is unknown but they do appear to agree with previous work showing polymorphs to have both a protective and destructive role in the pathogenesis of periodontal disease.

For both MPO activity and chemiluminescence, the association with 10^7/control bone loss gave a more definite pattern of results than with control bone loss. This suggests that certain differences of polymorph function between genotypes may only be disclosed following bacterial challenge. A number of previous

investigations have a bearing on this hypothesis.

Lamster et al (1980) examined mouse polymorphonuclear leucocyte-rich (PMNLr) cells, produced as a result of intraperitoneal injection of either 2 ml 1% glycogen in sterile saline (g cells) or 2 ml thioglycollate (th cells). The metabolic activity of both g and th cell groups was determined by measuring glucose utilization in the hexose monophosphate shunt. It was found that g cells had significantly lower metabolic activity and could depress both primary cell-mediated and secondary humoral immune responses. Conversely, th cells had high metabolic activity and stimulated both these responses. The mechanisms by which inflammatory cells modify the immune response is not definitely known, but a number of salient points have emerged. Nakayama et al (1978) showed that PMNLr cells could enhance cytolysis of tumour cells by allogeneic spleen cells, but that PMNLr cells alone had no effect. It was thought that some factor or factors released by PMNLr cells was responsible for the activity observed.

A variety of neutral proteases released from polymorphs have been shown to enhance immune function (Nakamura et al, 1976; Schmidt et al, 1978; Havemann et al, 1978; Bretz, 1978). The release of these enzymes seems to be correlated with metabolic activation of the polymorphs (Murphy, 1976b). Therefore, the stimulatory effects of the th cells in the study by Lamster et al (1980) could be at least partially accounted for by protease activity. The suppressive effect of the g cells in the same study may be attributable, as pointed out by the authors, to a different

polymorph released substance such as prostaglandin (PGE). Human polymorphs have been shown to release PGE1 when exposed to zymosan (Zurier & Sayadoff, 1975) and PGE is released from rabbit peritoneal polymorphs during phagocytosis (McCall & Youlten, 1973). The difficulty encountered in studying the effects of PGE in vivo relates to its relatively short half life. Strom et al (1977), however, have demonstrated that PGE derivatives, which have a longer half life, could suppress both in vivo and in vitro immune responses to alloantigens, causing prolongation of kidney allografts in rats, and reduction of in vitro lymphocyte-mediated cytotoxicity in mice.

The findings of Lamster et al (1980) argue against the interaction of polymorphs and immune cells as being specific for B cells, as had been proposed (Vischer et al, 1976; Bretz, 1978), since both lymphocyte-mediated cytotoxicity and complement-dependent cytotoxicity were modified by the acute inflammatory cells. The basic question of why different stimulants recruit inflammatory cells with different spontaneous metabolic activity has not been answered, but this finding underlines the importance of making sure all cells for comparative testing are treated in the same way.

6.4.4 CONCLUSION

The observed differences of polymorph function go some way towards explaining why different levels of bone loss, both naturally occurring and in response to A. viscosus inoculation, were found in the different genotypes.

Although no record of murine polymorph MPO activity or chemiluminescence on challenge with A. viscosus has appeared in the literature, Baron & Proctor (1984), using chemiluminescence as a measure of the response of BALB/c peritoneal exudate polymorphs to Salmonella typhimurium, showed that both polymorphs and specific anti-Salmonella antibodies acting in concert were required to effect polymorph-associated bactericidal activity. These authors also showed that polymorph activity alone does not play a major role in protecting unvaccinated, sensitive mice from disease caused by this organism. This again leads to the suggestion that the humoral immune system is extremely important in the murine response to bacterial challenge.

Both the MPO and chemiluminescence findings have thus generated interesting ideas, but proving or disproving them is not possible without performing polymorph function tests using either whole A. viscosus cells, or an extract thereof, as the stimulant. Such tests could also be performed on the cells of animals inoculated with 10^6, 10^7 and 10^9 cfu A. viscosus, to investigate possible differences in cellular function between these groups.

C H A P T E R 7

LYMPHOCYTES

7.1 I N T R O D U C T I O N

Investigations into the role of lymphoctyes in the pathogenesis of periodontal disease, from a number of points of view and using different techniques, have resulted in a mass of information, much of which is contradictory. However, these cells, with their potent immunoregulatory functions, are clearly a most important component of the host defence system.

7.1.1 THE BLASTOGENIC RESPONSE

The blastogenic response is an increase in lymphocyte numbers through cell division following some form of stimulation. In a series of reports, Ivanyi and Lehner (1970; 1971a,b; 1974) showed that cultures of peripheral blood mononuclear cells from patients with gingivitis and mild or moderate periodontitis, incubated in vitro with homogenates of a panel of bacteria from periodontal pockets or supragingival microbial plaque, exhibited blastogenesis whereas those from disease-free control subjects did not. By contrast, cells from patients with severe periodontitis did not respond blastogenically, although lymphokine production was found to be increased. These are soluble factors, derived from lymphocytes, which function as mediators of immune reactions (Ivanyi et al, 1972). Ivanyi et al (1973) determined that patients with severe disease carried serum factors which blocked the blastogenic response, while serum from patients with chronic gingivitis or mild to moderate periodontitis contained factors which enhanced the blastogenic response. These factors appeared to be immunoglobulins specific to the antigen activating the mononuclear response.

Church and Dolby (1978), in contrast to previous workers, examined the relationship between the dose of dentogingival plaque and the *in vitro* lymphoproliferative response in periodontally diseased individuals. The results of this work suggested that lower concentrations of plaque are needed to produce peak response values in severely diseased patients, implying that plaque elimination is more important for severely diseased individuals than for their less affected counterparts.

A positive correlation between the degree of periodontal disease as measured by the Russell Index (Russell, 1956) and spontaneous cell-mediated cytotoxicity ($P < 0.001$) was reported in 1985 by Tsoumis et al. The *in vivo* significance of this is obscure, but patients with Chediak-Higashi syndrome, who often suffer rapidly progressive periodontal disease (Tempel et al, 1972), have impaired cell-mediated cytotoxicity (Katz et al, 1982). Apparently, cell-mediated cytotoxicity is influenced by and may play a role in oral disease.

It is possible that HLA type is important in determining lymphocyte responsiveness, as Amer et al (1986) demonstrated impaired lymphocyte responsiveness to phytohaemagglutinin in HLA-B8 and/or DR3 subjects.

7.1.2 THE ROLE OF T-CELLS

In the early 1970's, a blastogenic response to antigen preparations by peripheral blood mononuclear cells in culture was considered to be an *in vitro* correlate of a delayed hypersensitivity reaction and only T-cells were thought to produce lymphokines. Thus, an important role for T-lymphocytes and

delayed hypersensitivity in the pathogenesis of chronic gingivitis and periodontitis was postulated (Page & Schroeder, 1982). This idea was supported by the observation of Ivanyi and Lehner (1977) that levamisole, a drug which stimulates cell-mediated immune responses, resulted in the development of gingivitis and aggravated the severity of established chronic gingivitis. Work by Wilde et al (1977) further supported this hypothesis in showing that typical early lesions in both rat and monkey gingivae could be induced by placing antigen at the gingival margin after sensitising the animals' skin to the same antigen. Sensitisation could also be transferred from one animal to another by lymphocytes and not by serum.

However, Mackler et al (1974) demonstrated that plaque bacterial products caused both B and T-cell activitation and that, of the two cell types, B-cells were the better lymphokine producers. Furthermore, the histological studies of Seymour and Greenspan (1979) and of Mackler et al (1977; 1978a,b; 1979) showed periodontitis to be characterised by a predominance of B-cells and plasma cells rather than T-cells and macrophages, suggesting a prominent role for B-cells.

7.1.3 INTERACTIONS BETWEEN T AND B-CELLS

The interrelationships of different cell types in periodontal disease have recently been studied by Baker and Tondreau (1985). These investigators, using peripheral blood lymphocytes from patients with 'moderate' disease only, examined the proliferative responses of B and T-cell populations, separately and in combination. The lymphocytes were exposed to

cell walls from seven types of oral bacteria to determine the antigenic response, and to phytohaemagglutinin (PHA), pokeweed mitogen (PWM), lipopolysaccharide (LPS) and streptolysin-O (SLO) to determine the mitogenic response. They found that mononuclear cells, together with a recombined subpopulation consisting of four parts purified T-lymphocytes and one part B-lymphocytes, responded significantly to all the stimulants. Purified T-lymphocytes alone responded significantly to PHA and PWM, but were unresponsive to oral bacteria and SLO until cultured with 2% autologous macrophages, indicating that the T-cell responses to oral bacteria were macrophage dependent. B-cells alone were poorly responsive to all the stimulants, but the responses to all stimulants except PHA increased significantly after the addition of 10% mitomycin-C-treated T-cells, showing that B-cell proliferation in response to these stimulants was T-cell dependent.

The dependence of T-cell proliferative responses to oral bacteria on macrophages had previously been reported by Stashenko (1982) who also found that 10% macrophage concentrations gave maximum T-cell proliferation while concentrations in excess of 20% were suppressive.

Many B-cell mitogens, including PWM (Levinson et al, 1983), LPS (Miller et al, 1978; Lopatin et al, 1980a), staphylococcal peptidoglycan (Levinson et al, 1983) and Actinomyces viscosus (Lopatin et al, 1980 b & c) have been shown to require T-cell help in order to stimulate optimal human B-cell proliferation in vitro. The recent work of Baker and Tondreau (1985) confirmed these earlier findings. The evidence as a whole, therefore, suggests

that examination of one subpopulation of lymphocytes alone cannot provide all the answers to questions about lymphocyte function in periodontal disease.

7.1.4 IS GINGIVAL LYMPHOCYTE ACTIVATION ANTIGENIC OR MITOGENIC?

If gingival lymphocytes are activated specifically by bacterial antigens (monoclonal activation) rather than non-specifically (polyclonal activation), specific antibody would be produced in diseased gingival tissues and immune complex deposition would occur. Such complexes can activate complement and so could interact with polymorphs and monocytes to initiate and perpetuate tissue destruction. If such specific sensitisation is reflected by lymphoid cells in the blood, responsiveness of peripheral blood mononuclear cells to plaque related antigens should vary with the periodontal disease status of the cell donor. On the other hand, if lymphocyte activation is polyclonal large amounts of nonspecific immunoglobulin would be produced, with little or no immune complex formation. It should then be unlikely that host disease status would be reflected in responsiveness of peripheral blood mononuclear cells. It must be noted, though, that both mitogenically (polyclonally) activated cells and their antigenically (monoclonally) activated counterparts produce lymphokines which may be key factors in tissue destruction (Page & Schroeder, 1982).

At a cellular level, several studies have shown that T-cell poliferative responses to mitogens are less macrophage-dependent than T-cell proliferative responses to antigens (Cline & Swett,

1968; Levis & Robbins, 1977; de Vries et al, 1979). The purified T-cells in the Baker and Tondreau study (1985) responded relatively well to the mitogens PHA and PWM in the absence of macrophages, although addition of macrophages did enhance the responses. The same T-cells were poorly responsive to the antigen SLO and oral bacteria and these responses were highly dependent on the presence of macrophages. This finding suggests that T-cell responses to oral bacteria are antigenic rather than mitogenic. On the other hand, many oral bacteria are known to activate polyclonal immunoglobulin production in human peripheral blood B-lymphocytes (Smith et al, 1980; Bick et al, 1981; Mangan et al, 1983). Furthermore, A. viscosus has been shown to activate polyclonal DNA synthesis in human B-cells, provided there is a source of activated T-cells (Lopatin et al, 1980b,c; 1981). Engel and co-workers (1977) demonstrated that A. viscosus homogenate contained substance(s) which caused spleen cells from conventional and germfree mice to undergo increased DNA synthesis. This mitogenic effect was apparently exerted primarily on B-cells as spleen cells from nude mice or T-depleted spleen cells from conventional mice responded as strongly as conventional (T + B) spleen cells and mouse thymocytes did not respond at all.

These findings were confirmed by Burckhardt et al (1977) who found that an extracellular heteroglycan and a sonicated cell supernatant of A. viscosus were mitogenic to mouse B-cells. The next step was to find, isolate and purify the mitogenic fraction(s).

Kimura et al (1983) isolated a murine B-cell mitogenic

component from both A. viscosus and A. naeslundii cell walls. However, Halfpap et al (1985) extracted various cell components from two strains of A. viscosus and tested the response of murine splenocytes to each one. They found that the mitogens produced by A. viscosus appeared to be either associated with whole cells and released by physical methods, such as passage through the French Pressure Cell, or were released into the medium during growth. The data strongly suggested that the mitogenic activity was not dependent on a major cell wall component because the most active fractions were soluble macromolecules released by physical methods. Such soluble preparations were substantially more active than were cell walls or peptidoglycan produced by any of the disruption methods used. Furthermore, complete solubilization of the cell walls with M-N-acetylmuramidase according to the method of Brown et al (1982) did not release activity that might have been masked in the intact cell wall. It is possible that the absence of mitogenic activity for A. viscosus peptidoglycan may be attributable to the unique structure of the peptidoglycan crossbridge as described by Schleiffer and Kandler (1972). In conclusion, Halfpap et al (1985) suggested that subsequent efforts in trying to identify murine B cell mitogens from A. viscosus should be directed towards components that could be sheared from the cells by the forces generated by passage through the French Pressure Cell under conditions in which cell lysis is minimal.

This evidence that B-cell proliferative responses to oral bacteria may be mitogenic still leaves unanswered whether T-cell proliferation and T-helper activation occur antigenically or

mitogenically. According to Baker and Tondreau (1985), a study using cord-blood lymphocytes is planned. Such cells are thought to be immunologically naive and, even though earlier studies (Horton et al, 1972; 1976; Baker et al, 1976; Chen & Doroszczak, 1980) failed to detect a proliferative response in them to oral bacteria, work by Donaldson et al (1983) showed that this lack of responsiveness could be overcome by altering the cell culture density.

Some authors (Chen & Doroszczak, 1980; Lopatin et al, 1980a; Stashenko, 1982) have pointed out that the kinetics of human peripheral blood lymphocyte DNA synthesis in response to oral bacteria, resembles an antigenic rather than a mitogenic response. DNA synthesis is maximal after six or seven days of culture, both for specific antigens and for oral bacteria, whereas mitogens such as PHA give maximal DNA synthesis after three days in culture. However, a six to seven day maximal DNA synthesis response is also typical of human lymphocyte responses to T-cell dependent B-cell mitogens such as LPS (Miller et al, 1978) and Staphylococcus aureus peptidoglycan (Dziarski et al, 1980). Consequently, kinetic studies are not reliable criteria for distinguishing between antigenicity and mitogenicity. Thus, at present, the antigenic versus mitogenic activation question remains unresolved.

7.1.5 THE PERIPHERAL MONOCYTE BLASTOGENIC RESPONSE AND PERIODONTAL DISEASE

Numerous investigators have repeated and extended the original experiments of Ivanyi and Lehner (1970). The first of

these were Horton et al (1972) who found a positive correlation between the peripheral monocyte blastogenic response to substances from microbial plaque and the presence/severity of gingivitis and periodontitis. A similar positive correlation was reported by Baker et al (1976) and additional confirmatory observations were reported by Horton et al (1973, 1976), Patters et al (1976), Lang & Smith (1977) and Smith & Lang (1977). Kiger et al (1974) found no correlation between blastogenesis and disease status but there was a positive correlation between the capacity of a patient's cells to respond to plaque bacterial preparations and their capacity to respond to other non-plaque antigens, thus suggesting that responsiveness is an individual characteristic unrelated to the presence of inflammatory periodontal disease.

Another approach has been to study cell responsiveness in subjects with experimental gingivitis. Lehner et al (1974) found a positive correlation between mononuclear cell responsiveness and the onset and severity of inflammation. The data have been judged inconclusive as the increase was small and the variance large. There were positive responses in this study to a purified protein derivative and Lactobacillus acidophilus, two stimulants not in any way aetiologically related to gingivitis, and this was taken to imply that plaque accumulation in humans has an adjuvant effect in that it may enhance immune responsiveness to all antigens.

Patters et al (1977, 1979) assessed mononuclear cell blastogenic responsiveness during a 12-week period of intensive tooth cleaning followed by a period of developing gingivitis. While no change was detected in PHA induced blastogenesis, the

stimulation index scores dropped in response to A. viscosus and F. nucleatum during tooth cleaning but rose in six out of eight subjects during the period of developing gingivitis. Lang and Smith (1976), using blood cells from patients developing gingivitis, found that the maximum response to PHA rose approximately four-fold and the dose required to obtain maximum blastogenesis dropped from 5 $\mu g\ ml^{-1}$ to 1 $\mu g\ ml^{-1}$. Responsiveness returned to normal after reinstitution of oral hygiene measures.

Suzuki et al (1984) presented data comparing 16 healthy controls and 20 periodontally diseased subjects. After a full clinical and radiographic examination of each patient, mixed lymphocyte cultures from peripheral blood were set up with homogenised cultures of B. gingivalis, F. nucleatum or A. viscosus, or with the mitogens LPS or PWM as stimulators. A range of cell and activator concentrations and incubation times was used. Under these conditions, cultures of activated patient cells did not differ significantly from activated control cells with any of the bacterial preparations. However, responsiveness of cultures from diseased subjects activated with both PWM and LPS was significantly lower than that of cultures of control cells.

Stashenko et al (1983) compared the clinical findings and cellular responsiveness of 33 diseased subjects and 11 healthy controls. The cell cultures used were T-cells plus 10% monocytes only and were tested against a panel of sonicates of 12 species of microorganism, including A. viscosus, and PHA. No statistically significant differences were found between the pooled results from

diseased subjects and those from controls. When all the subjects were grouped according to their T-cell response profile by cluster analysis, 'generalised high responder' and 'generalised low responder' groups were identified. Low responders were found to have significantly more redness and bleeding on probing than high responders. No differences were found, however, with respect to plaque, attachment level, pocket depth or bone loss. Patients not falling into the high or low responder groups had intermediate proliferative responses and moderate amounts of inflammation.

These apparently conflicting findings make the role of the blastogenic response in periodontal disease difficult to assess.

Lymphocyte studies have also been performed to try to shed some light on the role of the immune response in the control of collagen turnover. In 1982, Mammo et al showed that the cellular immune response to Type 1 collagen was higher in patients with periodontal disease than in their non-diseased counterparts. The work of Johnson and Ziff (1976) and Wahl et al (1978) showed that activated lymphocytes are capable of producing fibroblast stimulating factors while Anastassiades and Wood (1981) showed such cells to produce fibroblast inhibiting factors. Al-Dallal et al (1985) demonstrated the supernatants of mixed lymphocyte cultures to contain mediators with opposing in vitro growth effect on gingival fibroblasts at different times during culture. This last work thus supports the dual regulation shown by the findings of the previous three authors. Postlethwaite et al (1984) reported a similar phenomenon in relation to collagen production by fibroblasts.

It is not surprising, therefore, that this area of study is still regarded as largely mysterious and uncertain territory.

7.1.6 THE PRESENT STUDY

In the present study, experiments were designed to assess the response of both splenic and mesenteric lymph node (MLN) lymphocytes to both specific stimulation by the EDTA A. viscosus extract and mitogenic stimulation by PHA. The splenic cells were chosen to monitor the systemic immune system response and MLN cells to monitor the response of the gut-associated immune system. The effect of macrophage suppression on the different cell/stimulation combinations was tested by the addition of indomethacin and vitamin E which block any such suppression (deShazo et al 1981).

7.2 MATERIALS AND METHODS

The material for lymphocyte studies was obtained from approximately 10 mice of each genotype/inoculation group 12 weeks after inoculation with A. viscosus, according to the schedule described in Section 2.4).

7.2.1 LYMPHOCYTE TRANSFORMATION

Immediately postmortem, spleens and MLNs were dissected out in a laminar flow hood (Envair UK Ltd, Rossendale, UK) and immediately placed in sterile RPMI 1640 Dutch modification with Hepes medium (1640) (Flow Laboratories Ltd, Rickmansworth, UK). Tissues of each type from mice of the same genotype/inoculum group were then pooled.

The spleens were chopped finely in a sterile tissue culture dish with a sterile disposable blade. Using a fresh sterile disposable pipette for each group, 2 ml sterile 1640 was added and the tissue and medium mixed to release the lymphocyte rich cell population. The suspension was made up to 10 ml in a sterile centrifuge tube with fresh 1640 and allowed to settle for 5 minutes. All tube contents except the solids at the bottom of the tube were then layered over 5 ml premixed Ficoll Hypaque (Flow Laboratories Ltd, Rickmansworth, UK) in a fresh sterile disposable centrifuge tube. The tubes were then centrifuged at 200 g for 10 minutes. The supernatant, containing lymphocyte rich leucocytes, was removed immediately and washed twice in 1640 and the pellet, composed primarily of red blood cells, was discarded.

The MLNs were carefully trimmed free of fat, chopped finely

and resuspended in the manner described for spleens. The suspension containing the lymphocyte rich leucocytes was then washed twice in 1640. Pooled cells from each source were then counted in a 0.1 mm depth Neubauer Cell Counting Chamber (Weber-Werkzueg K G, Steinheim, West Germany) and cell viability was assessed by trypan blue exclusion. All cell suspensions were then adjusted to an appropriate working concentration as described below.

Spleen and MLN cells were cultured in sterile 96-well flat-bottomed microtitre culture plates (Flow Laboratories Ltd, Rickmansworth, UK). The cultures, in triplicate, contained 2 x 10^5 cells in a final volume of 0.2 ml consisting of 1640 supplemented with final concentrations of 5% fetal calf serum, 2 mM L-glutamine, 100 U ml^{-1} penicillin, 100 μg ml^{-1} streptomycin (all from Flow Laboratories, Rickmansworth, UK), and 5 x 10^{-5} M mercaptoethanol (Sigma Chemical Company, Poole, UK). For each genotype, cells were used either purely from one inoculum group or as 50/50 co-cultures from two inoculum groups. Co-cultures were designated, for example, as $10^6/10^7$.

Lymphocyte proliferation was induced by PHA (Sigma Chemical Company) or by the A. viscosus EDTA antigen extract (2.2.3.5), both at a final concentration of 5 μg ml^{-1}. Cultures were incubated at 37°C in a humidified atmosphere of 5% CO_2 in air. Incubation continued for three days for the mitogen (PHA) tests and six days for the antigen (A. viscosus) tests, with 0.5 μCi ^3H-thymidine GA (Amersham International plc, Amersham, UK) added to each well for the last 24 h of incubation. The cell cultures from each well were harvested using a cell harvester (Titertek,

Flow Laboratories Ltd, Rickmansworth, UK). The filters containing the harvested cells were then immersed in vials containing 3 ml scintillation fluid (Fisons Scientific Apparatus, Loughborough, UK) and the radioactivity counted in a liquid scintillation counter (Packard Tri-Carb Ltd, Caversham, UK). Readings were expressed as counts per minute (cpm).

Experiments with cells from different genotypes were performed on the dates given below. Only on 22.1.86 were cells of two different genotypes collected on the same day.

Genotype	Date
BALB/c	4.5.85, 8.1.86, 12.1.86, 22.1.86
C57	13.4.85, 21.9.85
+/bg	3.10.85
bg/bg	21.11.85, 22.11.85
ob/ob	27.4.85
CBA	22.1.86
xid/Y	10.1.86

7.2.2 MACROPHAGE MEDIATED SUPPRESSION

Microtitre lymphocyte cultures were set up as described above, except that each well also contained a final concentration of 50 g l^{-1} of both indomethacin and vitamin E (Flow Laboratories Ltd) abbreviated here as I/E. All cultures in triplicate were then incubated for the appropriate time according to whether stimulation was mitogenic or antigenic, with labelling, harvesting and counting as described above. Macrophage suppression would be indicated by increased blastogenesis in the presence of I/E.

7.3 R E S U L T S

In all cell preparations, viability was 98% or better as measured by trypan blue exclusion.

Tables including the number of observations, mean cpm and standard error for each group used in the analysis are given as an appendix to the main text of this chapter (Section 7.5).

7.3.1 DIFFERENCES BETWEEN RUNS ON DIFFERENT DAYS

For BALB/c, where the same lymphocyte transformation tests were repeated on four different days, one-way analysis of variance showed that for all 24 inoculum/cell/stimulation groups (uninoculated, 10^6, 10^7, 10^9/MLN, spleen/unstimulated, PHA, A. viscosus) that were tested on all four occasions, there were highly significant differences in blastogenic response between days ($P < 0.0001$ for 23 groups, $P = 0.004$ for one group).

7.3.2 DIFFERENCES BETWEEN GENOTYPES

Owing to the differences between runs for the same genotype (BALB/c) on different days, strictly valid comparison between different genotypes tested on different days could not be made. The only genotypes tested on the same day were BALB/c and CBA (on 22.1.86). Of the comparisons that could be made between these two genotypes within inoculum/cell/stimulation groups (uninoculated, 10^6, 10^7, 10^9/MLN spleen/unstimulated, PHA, A. viscosus) - all with and without I/E combined) eight showed a statistically significant difference between genotypes with CBA always having the higher blastogenic response ($P < 0.05$ by one-way analysis of variance).

Although comparison between genotypes tested on different

days is not strictly valid, an overall relationship between xid/Y, CBA and the other genotypes emerged. Of the 24 inoculum/cell/stimulation groups (uninoculated, 10^6, 10^7, 10^9/MLN, spleen/unstimulated, PHA, A. viscosus), xid/Y showed the lowest blastogenic response in 19 and CBA the highest in 13.

7.3.3 DIFFERENCES BETWEEN INOCULUM GROUPS

One-way analysis of variance failed to reveal any consistent pattern of differences between inoculum groups within genotypes, cell-types and stimulants (with and without I/E combined). The inconsistency of the results is illustrated by the testing of BALB/c cells from uninoculated controls and 10^7-inoculated animals on four different days. Considering only 8.1.86 (Table 7.1) there appears to be a difference between inoculum groups that is dependent on the source of the cells. However, this was not confirmed by results on the other three days.

7.3.4 DIFFERENCES BETWEEN MLN AND SPLEEN CELLS

Spleen cells tended to show a higher blastogenic response than MLN cells in unstimulated cultures, while in both PHA and A. viscosus stimulated cultures, with the exception of +/bg, MLN cells tended to show a higher response (Table 7.2).

7.3.5 DIFFERENCES BETWEEN STIMULANTS

Stimulation with PHA or A. viscosus resulted in significantly higher blastogenic responses than in unstimulated cultures for a large proportion of genotype/inoculum groups. PHA was a more potent stimulant for BALB/c, bg/bg, ob/ob, CBA and xid/Y, while A. viscosus was a more potent stimulant for C57 and +/bg (Table 7.3).

7.3.6 MACROPHAGE SUPPRESSION

Of the 32 statistically significant differences between cultures with and without I/E (Table 7.4), six showed a lower blastogenic response and 26 a higher blastogenic response with I/E, a significant preponderance of higher responses (chi-squared = 12.5, df = 1, $P < 0.001$). Significantly higher responses with I/E occurred predominantly with A. viscosus stimulation and in bg/bg mice. Twenty three of the 26 significantly higher responses occurred in mice with the C57 background.

7.3.7 CO-CULTURES

Statistically significant differences of blastogenic response between co-cultures and both their component cultures are summarised in Table 7.5. Of the 33 comparisons of $10^6/10^7$ co-cultures with both 10^6 and 10^7 pure cultures, four showed significantly higher responses. Of the 33 comparisons of $10^6/10^9$ co-cultures with both 10^6 and 10^9 cultures, only one showed a significantly higher response. There was no instance of a co-culture showing a significantly lower blastogenic response than both its component cultures.

Table 7.1 Summary of statistically significant differences of blastogenic response between uninoculated and 10^7 inoculated BALB/c mice on different days (with and without I/E combined).

	MLN			Spleen		
	Unstimulated	PHA	A. vis	Unstimulated	PHA	A. vis
4.5.85	-	$10^7 >$ Uninoc	-	-	$10^7 <$ Uninoc	$10^7 <$ Uninoc
8.1.86	$10^7 <$ Uninoc	$10^7 <$ Uninoc	-	$10^7 >$ Uninoc	$10^7 >$ Uninoc	$10^7 >$ Uninoc
12.1.86	-	-	-	$10^7 <$ Uninoc	-	$10^7 <$ Uninoc
22.1.86	-	-	-	-	-	$10^7 <$ Uninoc

Table 7.2 Differences in blastogenic responses: MLN - Spleen (with and without I/E combined). $P < 0.05$

		Unstimulated		PHA		A. viscosus	
		t	P	t	P	t	P
BALB/c	Uninoculated	-2.79	**0.008**	3.78	**0.000**	-0.55	0.586
	10^6	-3.24	**0.002**	0.50	0.620	1.01	0.318
	10^7	-2.72	**0.009**	0.12	0.901	0.39	0.702
	10^9	-2.06	**0.043**	3.99	**0.000**	2.24	**0.031**
	$10^6/10^7$	-4.78	**0.000**	-2.56	**0.018**	0.74	0.466
	$10^6/10^9$	-1.85	**0.069**	-1.08	0.289	0.57	0.573
C57	Uninoculated	-3.11	**0.005**	1.64	0.141	-2.90	**0.008**
	10^6	-0.72	0.477	-1.17	0.253	-0.50	0.631
	10^7	0.40	0.700	0.83	0.433	1.64	0.131
	10^9	1.69	0.109	0.90	0.393	2.88	**0.015**
	$10^6/10^7$	2.16	**0.046**	0.71	0.483	2.83	**0.017**
	$10^6/10^9$	1.96	0.069	0.48	0.638	3.01	**0.012**
+/bg	Uninoculated	-	-	-	-	-	-
	10^6	-6.44	**0.000**	-18.25	**0.000**	-1.01	0.338
	10^7	-3.79	**0.003**	-7.29	**0.000**	-0.41	0.687
	10^9	-5.83	**0.000**	-9.20	**0.000**	-2.34	**0.044**
	$10^6/10^7$	-	-	-	-	-	-
	$10^6/10^9$	-	-	-	-	-	-
bg/bg	Uninoculated	-1.09	0.287	7.91	**0.000**	1.54	0.154
	10^6	0.39	0.703	10.05	**0.000**	3.33	**0.008**
	10^7	-0.94	0.357	8.94	**0.000**	2.25	**0.048**
	10^9	-1.99	0.060	1.36	0.238	3.64	**0.019**
	$10^6/10^7$	1.25	0.244	6.01	**0.001**	4.26	**0.002**
	$10^6/10^9$	0.21	0.838	25.54	**0.000**	5.96	**0.000**
ob/ob	Uninoculated	-2.08	0.064	7.98	**0.000**	3.11	**0.011**
	10^6	-	-	-	-	-	-
	10^7	-11.80	**0.000**	8.79	**0.000**	0.32	0.752
	10^9	-0.91	0.386	19.40	**0.000**	-0.94	0.388
	$10^6/10^7$	-	-	-	-	-	-
	$10^6/10^9$	-	-	-	-	-	-
CBA	Uninoculated	-	-	-	-	-	-
	10^6	-6.82	**0.000**	-0.12	0.910	-0.47	0.647
	10^7	-1.49	0.150	5.87	**0.000**	3.31	**0.008**
	10^9	1.20	0.241	7.36	**0.000**	6.86	**0.000**
	$10^6/10^7$	-1.90	0.071	5.20	**0.000**	1.74	0.112
	$10^6/10^9$	-2.11	**0.047**	4.99	**0.001**	0.56	0.629
xid/Y	Uninoculated	-0.45	0.668	-0.34	0.737	0.60	0.564
	10^6	-1.54	0.138	1.71	0.119	-0.41	0.692
	10^7	1.05	0.303	0.54	0.599	-1.03	0.387
	10^9	1.64	0.115	-1.74	0.112	-1.65	0.185
	$10^6/10^7$	1.95	0.064	1.18	0.291	0.51	0.621
	$10^6/10^9$	-2.11	0.063	-0.65	0.530	-0.05	0.965

Table 7.3 Differences in blastogenic response: types of stimulation (with and without I/E combined). $P < 0.05$

Genotype	Inoculum	MLN						SPLEEN					
		Unstim.- PHA		Unstim.-A.vis		PHA - A.vis		Unstim.- PHA		Unstim.-A.vis		PHA - A.vis	
		t	P	t	P	t	P	t	P	t	P	t	P
BALB/c	Uninoculated	- 8.97	**0.000**	- 5.03	**0.000**	4.47	**0.000**	- 3.34	**0.004**	- 4.29	**0.001**	- 0.30	0.768
	10^6	- 8.63	**0.000**	- 6.52	**0.000**	1.79	0.080	- 4.50	**0.000**	- 4.12	**0.001**	1.64	0.111
	10^7	- 6.65	**0.000**	- 6.54	**0.000**	2.01	0.051	- 3.55	**0.002**	- 4.41	**0.000**	1.42	0.169
	10^9	- 6.35	**0.000**	- 6.66	**0.000**	3.72	**0.001**	- 3.49	**0.003**	- 3.15	**0.005**	1.07	0.292
	$10^6/10^7$	- 9.83	**0.000**	- 5.08	**0.000**	3.06	**0.004**	- 6.81	**0.000**	- 4.05	**0.001**	4.84	**0.000**
	$10^6/10^9$	- 7.87	**0.000**	- 4.84	**0.000**	5.29	**0.000**	- 8.37	**0.000**	- 4.08	**0.001**	6.49	**0.000**
C57	Uninoculated	- 2.66	**0.022**	- 5.44	**0.000**	0.93	0.372	- 4.10	**0.002**	-10.52	**0.000**	- 6.07	**0.000**
	10^6	- 3.44	**0.004**	- 3.33	**0.007**	-2.43	**0.032**	- 3.24	**0.008**	- 8.02	**0.000**	- 4.39	**0.000**
	10^7	- 1.76	0.110	- 2.04	0.066	-1.87	0.091	- 2.32	**0.039**	- 4.72	**0.001**	- 3.31	**0.005**
	10^9	0.61	0.553	- 2.84	**0.018**	-2.97	**0.013**	- 5.70	**0.000**	- 7.48	**0.000**	- 3.23	**0.004**
	$10^6/10^7$	0.19	0.851	- 3.10	**0.010**	-3.12	**0.010**	- 3.12	**0.008**	- 4.32	**0.001**	- 3.45	**0.005**
	$10^6/10^9$	0.27	0.787	- 3.19	**0.009**	-3.22	**0.008**	- 3.94	**0.002**	- 6.09	**0.000**	- 3.78	**0.002**
+/bg	Uninoculated	-	-	-	-	-	-	- 5.45	**0.000**	-16.81	**0.000**	-11.50	**0.000**
	10^6	1.79	0.095	- 7.38	**0.001**	-7.41	**0.001**	- 4.60	**0.000**	- 6.55	**0.002**	- 5.38	**0.003**
	10^7	1.54	0.142	- 7.08	**0.001**	-7.10	**0.001**	- 3.99	**0.004**	-14.72	**0.000**	- 8.74	**0.000**
	10^9	- 0.13	0.896	- 3.12	**0.036**	-3.12	**0.036**	- 3.01	**0.012**	- 6.58	**0.002**	- 5.19	**0.002**
	$10^6/10^7$	-	-	-	-	-	-	- 5.10	**0.000**	-15.49	**0.000**	-10.78	**0.000**
	$10^6/10^9$	-	-	-	-	-	-	- 4.97	**0.000**	-15.67	**0.000**	-13.23	**0.000**

bg/bg/

Table 7.3 (Continued)

		MLN						SPLEEN					
		Unstim.- PHA		Unstim.-A.vis		PHA - A.vis		Unstim.- PHA		Unstim.-A.vis		PHA - A. vis	
Genotype	Inoculum	t	P	t	P	t	P	t	P	t	P	t	P
bg/bg	Uninoculated	-15.21	**0.000**	- 5.81	**0.003**	13.90	**0.000**	- 9.47	**0.000**	- 2.50	0.067	8.07	**0.000**
	10^6	-18.35	**0.000**	- 5.59	**0.003**	15.96	**0.000**	- 7.27	**0.001**	- 3.36	**0.004**	6.35	**0.002**
	10^7	-22.67	**0.000**	- 5.14	**0.004**	18.15	**0.000**	- 9.24	**0.000**	- 2.38	**0.075**	7.77	**0.000**
	10^9	- 2.14	0.085	- 5.25	**0.004**	1.60	0.142	- 5.45	**0.003**	- 3.02	**0.008**	4.62	**0.007**
	$10^6/10^7$	- 7.07	**0.000**	0.15	0.888	8.62	**0.000**	- 8.42	**0.001**	- 0.66	0.516	8.08	**0.001**
	$10^6/10^9$	-34.33	**0.000**	- 8.64	**0.000**	27.17	**0.000**	-11.01	**0.000**	- 3.68	**0.002**	8.27	**0.000**
ob/ob	Uninoculated	-35.87	**0.000**	- 7.53	**0.001**	10.41	**0.000**	- 8.23	**0.000**	- 4.48	**0.007**	4.26	**0.002**
	10^6	-	-	-	-	-	-	-	-	-	-	-	-
	10^7	-15.61	**0.000**	- 5.70	**0.002**	12.77	**0.000**	- 3.74	**0.014**	- 1.38	0.240	2.52	**0.031**
	10^9	-29.97	**0.000**	- 4.18	**0.009**	12.15	**0.000**	- 2.69	**0.038**	- 1.00	0.365	- 0.97	0.376
	$10^6/10^7$	-	-	-	-	-	-	-	-	-	-	-	-
	$10^6/10^9$	-	-	-	-	-	-	-	-	-	-	-	-
CBA	Uninoculated	-	-	-	-	-	-	-16.13	**0.000**	- 9.60	**0.000**	3.59	**0.005**
	10^6	-20.08	**0.000**	- 6.34	**0.002**	7.77	**0.000**	-10.15	**0.000**	- 2.99	**0.009**	5.06	**0.000**
	10^7	-15.12	**0.000**	- 7.91	**0.001**	7.31	**0.000**	-15.24	**0.000**	- 4.78	**0.000**	7.94	**0.000**
	10^9	-16.52	**0.000**	-12.83	**0.000**	7.70	**0.000**	- 8.88	**0.000**	- 3.50	**0.003**	5.49	**0.000**
	$10^6/10^7$	-13.74	**0.000**	- 4.84	**0.005**	6.62	**0.000**	- 8.36	**0.001**	- 3.16	**0.020**	4.49	**0.001**
	$10^6/10^9$	-26.73	**0.000**	- 9.92	**0.000**	17.56	**0.000**	-12.97	**0.000**	- 3.24	**0.028**	4.89	**0.001**
xid/Y	Uninoculated	- 2.82	**0.038**	0.11	0.916	2.83	**0.018**	- 3.78	**0.013**	2.04	0.058	3.90	**0.012**
	10^6	- 3.05	**0.029**	0.26	0.795	3.05	**0.012**	- 3.20	**0.006**	1.24	0.234	2.34	0.067
	10^7	- 3.53	**0.017**	1.47	0.161	3.56	**0.005**	- 5.29	**0.003**	- 0.84	0.472	5.18	**0.004**
	10^9	- 3.03	**0.029**	1.51	0.149	3.07	**0.012**	- 7.28	**0.001**	- 1.55	0.178	7.18	**0.001**
	$10^6/10^7$	- 2.73	**0.041**	1.28	0.226	2.75	**0.021**	- 4.27	**0.008**	- 0.44	0.668	4.25	**0.008**
	$10^6/10^9$	- 3.37	**0.020**	- 3.10	**0.007**	3.34	**0.008**	- 3.15	**0.025**	0.84	0.413	3.19	**0.024**

Table 7.4 Differences in blastogenic response: without I/E - with I/E. ($P < 0.05$)

Genotype	Inoculum	MLN						SPLEEN					
		Unstimulated		PHA		A. viscosus		Unstimulated		PHA		A. viscosus	
		t	P	t	P	t	P	t	P	t	P	t	P
BALB/c	Uninoculated	1.20	0.236	- 0.77	0.451	-1.27	0.218	- 0.04	0.971	- 1.25	0.252	- 1.21	0.244
	10^6	- 0.80	0.431	- 2.05	0.062	-2.17	**0.042**	0.61	0.547	- 1.13	0.275	- 0.95	0.357
	10^7	0.24	0.808	- 1.77	0.091	-1.20	0.241	1.33	0.201	- 0.34	0.735	- 0.83	0.417
	10^9	- 1.00	0.329	- 0.90	0.377	-0.89	0.382	1.10	0.278	- 1.05	0.307	0.29	0.778
	$10^6/10^7$	0.82	0.427	0.38	0.720	-0.34	0.742	0.26	0.797	0.12	0.903	0.07	0.949
	$10^6/10^9$	- 0.17	0.869	0.07	0.946	-0.21	0.837	- 1.40	0.170	1.19	0.252	- 1.02	0.324
C57	Uninoculated	0.07	0.947	1.38	0.258	3.49	**0.006**	0.62	0.544	- 3.59	**0.005**	- 2.64	**0.025**
	10^6	- 0.14	0.893	1.00	0.339	-0.33	0.747	- 1.00	0.367	0.35	0.734	- 2.79	**0.042**
	10^7	0.51	0.616	- 1.29	0.264	-1.06	0.313	0.67	0.515	- 1.52	0.159	- 1.65	0.151
	10^9	1.51	0.172	- 0.18	0.861	-0.44	0.668	2.29	**0.037**	- 0.64	0.537	- 0.79	0.483
	$10^6/10^7$	- 0.46	0.650	- 0.20	0.843	0.26	0.804	- 0.75	0.462	- 3.09	**0.011**	- 1.30	0.262
	$10^6/10^9$	- 0.71	0.510	0.60	0.562	0.09	0.928	- 0.25	0.807	- 0.23	0.820	- 0.16	0.874
+/bg	Uninoculated	-	-	-	-	-	-	0.19	0.854	- 4.15	**0.014**	1.81	0.145
	10^6	- 0.59	0.570	- 0.64	0.559	0.73	0.508	- 2.07	0.065	- 2.75	0.051	0.20	0.854
	10^7	- 1.02	0.332	- 1.58	0.259	1.09	0.338	- 0.21	0.837	- 1.47	0.214	- 0.39	0.713
	10^9	0.38	0.711	- 1.99	0.117	-21.94	**0.000**	0.64	0.538	5.08	**0.007**	0.16	0.880
	$10^6/10^7$	-	-	-	-	-	-	- 0.67	0.522	- 8.26	**0.001**	- 0.20	0.848
	$10^6/10^9$	-	-	-	-	-	-	0.25	0.806	- 0.87	0.434	1.68	0.169
bg/bg/													

Table 7.4 (Continued)

Genotype	Inoculum	MLN						SPLEEN					
		Unstimulated		PHA		A. viscosus		Unstimulated		PHA		A. viscosus	
		t	P	t	P	t	P	t	P	t	P	t	P
bg/bg	Uninoculated	- 0.85	0.413	- 0.98	0.382	-5.17	**0.007**	- 0.49	0.633	- 1.19	0.366	- 4.11	**0.015**
	10^6	- 1.04	0.324	0.17	0.823	-7.91	**0.001**	- 0.74	0.477	- 2.45	0.070	- 7.01	**0.002**
	10^7	0.44	0.670	- 0.93	0.403	-13.69	**0.000**	- 0.23	0.824	- 2.33	0.080	- 6.53	**0.003**
	10^9	1.85	0.093	12.52	**0.006**	-4.68	**0.009**	- 0.28	0.798	- 0.01	0.992	- 2.35	0.079
	$10^6/10^7$	- 1.71	0.149	- 0.90	0.417	-2.71	0.054	- 0.29	0.777	- 0.97	0.389	- 9.53	**0.001**
	$10^6/10^9$	- 1.95	0.080	- 0.92	0.408	-2.07	0.108	- 0.10	0.926	- 1.77	0.151	- 3.29	**0.030**
ob/ob	Uninoculated	4.80	**0.009**	- 1.12	0.325	- 3.48	0.097	13.31	**0.000**	- 8.27	**0.001**	- 3.66	**0.02**
	10^6	-	-	-	-	-	-	-	-	-	-	-	-
	10^7	- 3.56	**0.024**	- 1.53	0.201	- 0.32	0.763	0.11	0.919	- 8.76	**0.001**	- 2.83	0.110
	10^9	- 8.36	**0.001**	- 1.87	0.134	- 3.77	**0.020**	- 1.68	0.242	- 4.62	**0.010**	- 1.00	0.423
	$10^6/10^7$	-	-	-	-	-	-	-	-	-	-	-	-
	$10^6/10^9$	-	-	-	-	-	-	-	-	-	-	-	-
CBA	Uninoculated	-	-	-	-	-	-	- 0.19	0.850	- 2.25	0.088	- 0.45	0.677
	10^6	- 0.43	0.674	- 1.69	0.166	- 2.20	0.092	- 0.29	0.780	- 0.01	0.992	- 0.73	0.504
	10^7	- 0.99	0.344	- 0.32	0.767	0.27	0.803	- 1.08	0.305	- 0.38	0.724	- 2.12	0.101
	10^9	- 0.23	0.824	- 0.34	0.752	- 0.84	0.446	0.51	0.624	0.60	0.581	- 0.80	0.470
	$10^6/10^7$	- 0.67	0.517	- 0.04	0.972	- 0.96	0.393	2.03	0.070	- 0.51	0.638	- 2.77	**0.050**
	$10^6/10^9$	- 1.46	0.174	1.81	0.145	0.12	0.912	0.09	0.933	- 1.47	0.217	- 0.36	0.738
xid/Y	Uninoculated	0.26	0.813	- 1.90	0.130	- 2.70	0.117	0.52	0.614	- 1.14	0.317	- 2.40	0.074
	10^6	- 0.52	0.613	- 1.68	0.168	- 4.27	**0.013**	- 0.58	0.575	- 1.98	0.118	- 0.68	0.532
	10^7	- 0.48	0.644	- 1.05	0.351	- 1.20	0.297	- 0.54	0.598	- 1.47	0.215	0.81	0.463
	10^9	- 0.52	0.615	- 1.10	0.332	- 1.70	0.165	- 0.79	0.448	- 0.19	0.861	- 1.78	0.219
	$10^6/10^7$	0.72	0.486	0.11	0.914	2.04	0.112	- 0.67	0.516	0.28	0.795	- 2.42	0.073
	$10^6/10^9$	- 0.83	0.425	- 1.59	0.186	- 1.74	0.157	0.59	0.567	- 2.11	0.103	- 1.84	0.140

Table 7.5 Summary of statistically significant differences in blastogenic response between cultures (with and without I/E combined).

	MLN			Spleen		
	Unstimulated	PHA	A. vis	Unstimulated	PHA	A. vis
BALB/c	-	-	-	-	$10^6/10^7 > 10^6$ & 10^7 $10^6/10^9 > 10^6$ & 10^9	-
C57	-	-	-	-	-	-
+/bg	No data	No data	No data	-	-	-
bg/bg	$10^6/10^7 > 10^6$ & 10^7	-	-	$10^6/10^7 > 10^6$ & 10^7	-	-
CBA	-	$10^6/10^7 > 10^6$ & 10^7	-	-	-	-
xid/Y	-	-	-	-	-	-

7.4 D I S C U S S I O N

The high level of viability of cells indicates that the extraction procedures did not cause irreversible damage to either MLN or spleen cells. A disappointing feature of the results, however, was the difference between corresponding lymphocyte transformation tests for the same genotype on different days, making valid comparisons between runs on different days impossible. However, for each genotype, different inoculum groups, both cell types and all three stimulation groups (with and without I/E) were tested on the same day, so that comparisons between inoculum groups, cell types and stimulants within genotypes, as well as the assessment of macrophage suppression, were valid.

7.4.1 DIFFERENCES BETWEEN GENOTYPES

The only strictly valid comparison was that between BALB/c and CBA, where CBA showed a significantly higher blastogenic response than BALB/c. The reasons for this are not clear, but such differences are to be expected between inbred strains.

The generally low blastogenic response of xid/Y was consistent with the B-cell defect known to occur in this mutant. As discussed previously (2.1.2.3), xid/Y mice mount antibody responses to T-cell dependent antigens, thus suggesting that the T-cells are functionally operative. PHA is a T-cell mitogenic stimulant, and a detectable response would therefore be expected

in mixed cell populations. The reason for unresponsiveness in the majority of inoculum/cell/stimulation groups is not clear. It may, however, have been caused by lack of recognition of the stimulants, or T-cell function may not have been completely normal. It is apparent that this effect, by being present in all stimulation groups, is not A. viscosus mediated. In this genotype, in particular, study of separated B and T-cell populations would be valuable.

7.4.2 DIFFERENCES BETWEEN INOCULUM GROUPS

The fact that no consistent pattern of blastogenic response was associated with inoculum group, and thus with bone loss, can be interpreted in two ways. First, the function of the MLN and spleen cells, as measured by lymphocyte transformation, may not be of central importance in the pathogenesis of periodontal disease. Second, the tests themselves may not be accurate enough to detect relevant differences between inoculum groups, either because they were performed on mixed cell populations or because the techniques themselves were too crude.

7.4.3 DIFFERENCES BETWEEN MLN AND SPLEEN CELLS

The tendency for higher blastogenic responses by unstimulated spleen cells than by unstimulated MLN cells (Table 7.2) could have been caused by intrinsic differences between these two cell populations. This is supported by the finding that PHA stimulation tended to result in a higher blastogenic responses by MLN cells than by spleen cells. As PHA is a T-cell mitogen, it may be inferred that T-cells constitute a higher proportion of MLN than of spleen cells. It is possible, though, that the different cell

extraction procedures used for each organ type (Section 7.2.1) may favour the retention of one cell type rather than another.

The fact that A. viscosus stimulation also tended to produce higher blastogenic responses from MLN cells may be due to the following. Oral inoculation must have resulted in the swallowing of some proportion of the initial inoculum of A. viscosus. The antigens thus ingested would therefore first be processed by the gut-associated lymphoid tissue of which MLN cells are a constituent part. It is possible that the systemic immune system, as represented by the spleen cells, may only receive excess antigen 'spilled over' from the gut-associated immune system. If this was the case, even with an intrinsically higher blastogenic response of spleen as compared to MLN cells, the response to the antigenic challenge posed by A. viscosus extract might have been greater in the MLN cells as the antigen exposure would have been greater. The logical conclusion would be that, as the initial inoculum dose of A. viscosus increased, so the 'spill over' effect to the systemic immune system would increase, and thus the blastogenic response of spleen cells to A. viscosus stimulation would increase directly with initial inoculum dose. Unfortunately, evidence for this was not forthcoming but, as previously mentioned, this could have been caused by the experimental techniques themselves.

7.4.4 DIFFERENCES BETWEEN STIMULANTS

As discussed earlier in this chapter, PHA is T-cell mitogen whereas the A. viscosus extract was a mixture of non-covalently bound surface antigens. As expected, stimulation by

either of these tended to produce a higher blastogenic response than found in unstimulated cultures. A. viscosus extract induced a greater blastogenic response than PHA in both C57 and +/bg cultures (Table 7.3), but not in cultures from other genotypes. It is possible that these genotypes have an intrinsically greater B-cell population which responds to B-cell mitogens in the A. viscosus preparation. Alternatively, this could perhaps be ascribed to some unknown genetic difference and to the fact that the A. viscosus extract was more particulate in nature. It is possible that this difference in blastogenic response could have been associated with the C57 background, though neither ob/ob nor bg/bg showed the same effect. The presence of a homozygous mutant gene, however, may have overriden any influence of genetic background. For the other genotypes, BALB/c, bg/bg, ob/ob, CBA and xid/Y, where blastogenic responses were greater in response to PHA stimulation, the proportional contribution of T-cells may have been greater.

7.4.5 MACROPHAGE SUPPRESSION

Only a small proportion of comparisons between cultures with and without I/E showed statistically significant differences (Table 7.4). However, among these there was a significant preponderance of higher responses with I/E, especially with A. viscosus extract stimulation and in cultures from bg/bg mice. The findings therefore suggest that macrophage suppression does occur in the response to A. viscosus and that it is under genetic control.

7.4.6 CO-CULTURES

The co-culture experiments were included to detect T-cell suppression or help. The fact that no co-culture showed a significantly lower blastogenic response than those of the constituent pure cultures, and that five co-cultures showed a significantly greater response than those of their pure components, could be regarded as weak evidence of T-cell help (Table 7.5). This evidence, however, is based on only five results out of a total of 66. It is possible, therefore that, although statistically significant, these results may not have a biological basis.

7.4.7 GENERAL DISCUSSION

The results of the experiments described in this chapter may be summarised as follows.

(a) The inter-run variation in blastogenesis was too great to allow comparison of experiments performed on different days.

(b) No consistent differences between inoculum groups, and thus related to bone loss, were apparent.

(c) With no stimulation, there was a tendency for a higher blastogenic response in spleen cells than MLN cells. With stimulation by either PHA or A. viscosus the reverse was found. This supports the idea that A. viscosus inoculations led to greater stimulation of the gut-associated lymphoid tissue than the systemic immune system.

(d) The effect of each stimulant on blastogenesis seemed to be at least partly determined by genotype but the findings were unrelated to levels of induced periodontal disease.

(e) There was weak evidence for the presence of both T-cell help and macrophage suppression, although again this was unrelated to levels of induced periodontal disease.

In commenting on the somewhat inconclusive nature of the lymphocyte findings, it could be said that the source of cells used was not appropriate. However, although immunological aspects of the microenvironment of the gingival crevice are probably most relevant, this area in the mouse is too small to provide sufficient numbers of cells for experimentation. In any case, this does not obviate the value of examining immunocompetent cells representative of the systemic and gut-associated immune systems, as in vitro systems can be manipulated to favour relevant clones of cells.

Unseparated lymphocyte cultures, as used in the current work, contain a diversity of functionally distinct populations of immunocompetent cells, and proliferation is only an index of the product of numerous cell-cell interactions. Variation in the proportions of different cell populations could thus contribute to the variability of the results, even within triplicates treated in a similar fashion. Separation of lymphocyte cultures into their different cell component types was not performed, mainly through lack of time, as the removal and storage of serum and jaw dissections all had to be performed on the same day as the spleen and MLN dissections and cell preparation, counting and plating. The results are thus a composite of B and T-cell responses and it is not possible to ascribe greater importance to either group of cells. However, the results suggest that future study of separated B and T-cells would be valuable.

One of the most surprising results was the lack of positive evidence for the modulating effect of macrophages. This was unexpected as it is generally accepted that macrophages have a major role in the immune response, both as essential accessory cells and as regulatory cells that can either enhance or suppress immune reactions. Activated macrophages can suppress lymphocyte proliferation in vitro by prostaglandin and oxygen radical production (deShazo et al, 1981).

The current findings do not necessarily imply that macrophages are not important in periodontal disease. Mononuclear phagocytic cells (macrophages and monocytes) are present in normal and inflammed gingiva, both in human and animal periodontal disease (Zachrisson, 1968; Pearshall & Weiser, 1970; Payne et al, 1975). These cells arise from blood monocytes (Ebert & Florey, 1939) which are chemotactically attracted to the inflammatory site by numerous substances such as complement (Snyderman et al, 1972), bacterial products (Ward, 1968) and lymphokines (Ward et al, 1970), and then become activated tissue macrophages (Ebert & Florey, 1939).

It has been shown by Richman et al (1979) that A. viscosus activates macrophages in vitro. Such macrophages can phagocytose cellular debris that polymorphs cannot (Pearshall & Weiser, 1970) as well as synthesise and release lysosomal enzymes (Page et al, 1973; Wahl et al, 1974). It has been suggested that the breakdown of collagen during inflammation is a consequence of the release of lysosomally derived enzymes which activate collagenase (Houck et al, 1968). Other enzymes such as beta-glucuronidase or N-acetyl-

beta-galactosidase, could hydrolyse the mucopolysaccharides of the connective tissue ground substance and also the extracellular matrix of the epithelium. Furthermore, it has been suggested that macrophages could play a role in bone resorption (Khan et al, 1978) and that macrophage-derived cells could phagocytose collagen fibrils exposed after osteoclastic activity (Rifkin & Heijl, 1979). The presence of activated macrophages within inflammed gingiva would probably indicate an attempt to remove the irritants. On the other hand, macrophages could participate in the destruction of the periodontium through the release of lysosomal enzymes during activation. As an extension of the current work, it would perhaps be worthwhile to investigate the macrophage presence in induced disease by cytochemical techniques such as those described by Charon et al (1981).

Fitzgerald et al (1981) detected a significant lymphoproliferative response to A. viscosus antigens in cervical node cells from animals inoculated with 10^7 cfu but not in cells from animals inoculated with 10^9 cfu. Stimulation with LPS, a B-cell mitogen, gave the same result, implying that B-cells were predominantly the responding cell type. This finding is not consistent with the results of the current work. Engel et al (1977) showed that A. viscosus homogenate contained substances which caused splenic lymphocytes from both germ-free and conventional mice to undergo blastogenesis. These workers also showed that the mitogenic effect was primarily directed to B-cells, as spleen cells from nude mice, or T-depleted spleen cells from conventional mice, responsed as strongly as a normal mixture

of T and B-cells. T-cells alone did not respond mitogenically to A. viscosus. Fitzgerald and Birdsell (1982) reported similar results, suggesting a predominant B-cell response in 10^6 and 10^7 inoculated animals as compared to controls. These authors suggested that either the proportion of B-cells increases in draining lymph nodes and spleen of infected animals, or that the organs of infected mice contained a larger number of B-cells which were able to proliferate better in response to LPS than cells from uninoculated mice. It has been shown by Gronowitz & Couthino (1975) that different types of B-cell mitogen exist. Dextran sulphate acts on immature cells to turn them into pre-antibody producing intermediate cells while LPS acts on intermediate cells to turn them into antibody producing mature cells. It is possible that A. viscosus inoculations may induce immature B-cells to change into intermediate stage B-cells which are more LPS responsive. This could account for the higher response observed with LPS without postulating an increase in the proportion of B-cells. It is generally accepted that B-cells and plasma cells are the dominant cell types found in periodontally diseased tissue.

Fitzgerald and Birdsell (1982) also showed that concanavalin A (ConA) stimulation of cells from both 10^6 and 10^7 inoculated mice resulted in a greater blastogenic response than such stimulation of control cells which showed little or no response. Thus, because ConA is a T-cell mitogen, the implication is that A. viscosus inoculation affected T-cells also, leading Fitzgerald and Birdsell (1982) to conclude that A. viscosus non-specifically alters the immune system.

The current work adds little in terms of either opposition to or support for these previous findings. Further work is required and, if a lymphocyte study is to be seriously undertaken, T and B-cell separation must be performed. Perhaps recombination of these two cell types in different proportions would provide further information. So far, the role of T-cell regulation of B-cells has not been investigated and this could be done by in vitro immunoglobulin production experiments.

7.5 DESCRIPTIVE STATISTICS

Tables showing the number of observations(n), mean blastogenic response (cpm) and s.e. for each genotype/inoculum group.

Stimulation with and without I/E combined: MLN

		ZERO				PHA				A. VISCOSUS			
		n	Mean	±	s.e.	n	Mean	±	s.e.	n	Mean	±	s.e.
BALB/c	Uninoculated	41	1062	±	127	24	21197	±	2240	22	8838	±	1542
	10^6	40	1078	±	233	24	13629	±	1436	22	10066	±	1358
	10^7	41	997	±	165	24	12669	±	1748	24	8476	±	1132
	10^9	39	970	±	195	22	22451	±	3379	22	9098	±	1205
	$10^6/10^7$	35	617	±	109	18	15345	±	1495	18	8680	±	1583
	$10^6/10^9$	36	953	±	123	16	26327	±	3223	18	7736	±	1395
C57	Uninoculated	18	154	±	31	12	3420	±	1227	12	2228	±	380
	10^6	18	427	±	128	12	1493	±	282	12	4624	±	1255
	10^7	18	530	±	179	12	1812	±	708	12	16152	±	7654
	10^9	18	3929	±	2166	12	2566	±	568	12	42455	±	13415
	$10^6/10^7$	18	1517	±	509	11	1403	±	323	12	34799	±	10708
	$10^6/10^9$	18	1966	±	821	12	1715	±	403	12	35467	±	10483
+/bg	Uninoculated	-	-	-	-	-	-	-	-	-	-	-	-
	10^6	12	629	±	100	6	429	±	50	6	45657	±	6104
	10^7	12	508	±	56	6	368	±	59	6	50576	±	7073
	10^9	12	417	±	56	6	431	±	105	5	25680	±	8103
	$10^6/10^7$	-	-	-	-	-	-	-	-	-	-	-	
	$10^6/10^9$	-	-	-	-	-	-	-	-	-	-	-	-

bg/bg/

		ZERO				PHA				A. VISCOSUS			
		n	Mean	±	s.e.	n	Mean	±	s.e.	n	Mean	±	s.e.
bg/bg	Uninoculated	12	459	±	85	6	24038	±	1548	6	2169	±	282
	10^6	11	772	±	125	6	28200	±	1489	6	3372	±	448
	10^7	12	667	±	113	6	34100	±	1471	6	4402	±	718
	10^9	12	422	±	102	6	13156	±	5946	6	3618	±	600
	$10^6/10^7$	12	4581	±	2483	6	35105	±	3556	6	4208	±	432
	$10^6/10^9$	12	840	±	138	6	306266	±	857	6	4679	±	422
ob/ob	Uninoculated	6	102	±	25	6	16545	±	458	6	6499	±	849
	10^6	-	-	-	-	-	-	-	-	-	-	-	-
	10^7	6	326	±	31	6	19061	±	1200	6	2781	±	429
	10^9	6	1886	±	135	6	23086	±	694	6	6738	±	1152
	$10^6/10^7$	-	-	-	-	-	-	-	-	-	-	-	-
	$10^6/10^9$	-	-	-	-	-	-	-	-	-	-	-	-
CBA	Uninoculated	-	-	-	-	-	-	-	-	-	-	-	-
	10^6	12	5006	±	414	6	42986	±	1845	6	19952	±	2320
	10^7	12	4710	±	625	6	45789	±	2644	6	21462	±	2024
	10^9	12	7907	±	888	6	58714	±	2944	6	31545	±	1941
	$10^6/10^7$	12	6568	±	463	6	55032	±	3496	6	22954	±	3353
	$10^6/10^9$	12	6695	±	955	6	51101	±	1368	6	21786	±	958
xid/Y	Uninoculated	12	60	±	11	6	593	±	189	6	57	±	17
	10^6	11	52	±	7	6	2431	±	781	6	49	±	9
	10^7	12	67	±	7	6	1843	±	503	6	50	±	9
	10^9	12	65	±	7	6	1279	±	400	6	50	±	4
	$10^6/10^7$	12	67	±	7	6	1597	±	561	6	58	±	3
	$10^6/10^9$	12	37	±	2	6	1017	±	290	6	47	±	3

Stimulation with and without I/E combined: Spleen

		ZERO			PHA			A. VISCOSUS		
		n	Mean	± s.e.	n	Mean	± s.e.	n	Mean	± s.e.
BALB/c	Uninoculated	36	2002	± 312	20	9298	± 2159	18	10157	± 1877
	10^6	38	2364	± 326	20	12355	± 2196	20	8117	± 1358
	10^7	36	2063	± 356	21	12262	± 2851	20	7826	± 1256
	10^9	38	1748	± 324	20	7496	± 1616	20	5401	± 1114
	$10^6/10^7$	32	1667	± 191	17	24830	± 3396	18	7151	± 1339
	$10^6/10^9$	35	1296	± 140	18	31611	± 3620	16	6639	± 1303
C57	Uninoculated	18	413	± 77	12	1379	± 222	12	3622	± 295
	10^6	18	572	± 156	12	2117	± 451	12	5308	± 570
	10^7	18	450	± 95	12	1175	± 298	12	3527	± 645
	10^9	17	267	± 45	12	1991	± 299	12	3787	± 469
	$10^6/10^7$	18	398	± 99	12	1131	± 213	12	4377	± 916
	$10^6/10^9$	18	350	± 75	12	1482	± 277	12	3911	± 580
+/bg	Uninoculated	12	9675	± 1170	6	18366	± 1083	6	51164	± 2639
	10^6	12	10445	± 1521	6	18841	± 1008	6	54688	± 6584
	10^7	12	5827	± 1402	6	16061	± 2150	6	53899	± 3757
	10^9	12	10244	± 1685	6	17864	± 1891	6	47877	± 5465
	$10^6/10^7$	11	6403	± 1299	6	15107	± 1106	6	50762	± 3119
	$10^6/10^9$	12	7533	± 1481	6	15910	± 805	6	49955	± 2443

bg/bg/

		ZERO				PHA				A. VISCOSUS			
		n	Mean	±	s.e.	n	Mean	±	s.e.	n	Mean	±	s.e.
bg/bg	Uninoculated	12	595	±	92	6	9659	±	953	6	1483	±	343
	10^6	12	703	±	125	6	9208	±	1163	6	1600	±	288
	10^7	12	845	±	151	6	15101	±	1535	6	2306	±	594
	10^9	12	736	±	120	6	4995	±	772	6	1354	±	161
	$10^6/10^7$	12	1450	±	322	6	12420	±	1263	6	17998	±	364
	$10^6/10^9$	12	798	±	149	6	5966	±	445	6	1787	±	238
ob/ob	Uninoculated	6	230	±	56	6	8099	±	955	6	3166	±	653
	10^6	-	-	-	-	-	-	-	-	-	-	-	-
	10^7	6	1720	±	114	6	5424	±	984	6	2545	±	585
	10^9	6	2246	±	373	6	4316	±	674	6	90441	±	88587
	$10^6/10^7$	-	-	-	-	-	-	-	-	-	-	-	-
	$10^6/10^9$	-	-	-	-	-	-	-	-	-	-	-	-
CBA	Uninoculated	12	9676	±	847	6	35591	±	1366	6	27030	±	1954
	10^6	12	13473	±	1169	6	43365	±	2701	6	121851	±	3281
	10^7	12	6159	±	745	6	28576	±	1269	6	213176	±	1468
	10^9	12	6326	±	966	6	30212	±	2512	6	312968	±	1890
	$10^6/10^7$	12	8226	±	740	6	31928	±	2736	6	215871	±	2306
	$10^6/10^9$	12	9345	±	793	6	38566	±	2109	6	219935	±	3171
xid/Y	Uninoculated	12	66	±	5	6	679	±	162	6	46	±	10
	10^6	11	69	±	8	6	948	±	382	6	54	±	7
	10^7	12	56	±	8	6	1531	±	279	6	81	±	29
	10^9	12	51	±	6	6	2136	±	286	6	76	±	15
	$10^6/10^7$	12	50	±	6	6	896	±	198	6	54	±	7
	$10^6/10^9$	12	62	±	12	6	1341	±	405	6	48	±	7

Blastogenic response with and without I/E: MLN with zero stimulation

		Without I/E				With I/E			
		n	Mean	±	s.e.	n	Mean	±	s.e.
BALB/c	Uninoculated	21	1210	±	203	20	906	±	146
	10^{-}	20	890	±	223	20	1266	±	412
	10^{7}	20	1039	±	220	21	957	±	249
	10^{9}	18	769	±	178	21	1142	±	328
	$10^{6}/10^{7}$	18	703	±	194	17	526	±	95
	$10^{6}/10^{9}$	18	933	±	149	18	974	±	199
C57	Uninoculated	9	156	±	23	9	151	±	60
	10^{6}	9	409	±	174	9	445	±	198
	10^{7}	9	624	±	275	9	437	±	242
	10^{9}	9	7082	±	4166	9	775	±	321
	$10^{6}/10^{7}$	9	1276	±	603	9	1758	±	850
	$10^{6}/10^{9}$	9	1371	±	663	9	2561	±	1529
+/bg	Uninoculated	-	-	-	-	-	-	-	-
	10^{6}	6	569	±	110	6	690	±	174
	10^{7}	6	451	±	76	6	565	±	83
	10^{9}	6	439	±	99	6	395	±	60
	$10^{6}/10^{7}$	-	-	-	-	-	-	-	-
	$10^{6}/10^{9}$	-	-	-	-	-	-	-	-

bg/bg/

		Without I/E				With I/E			
		n	Mean	±	s.e.	n	Mean	±	s.e.
bg/bg	Uninoculated	6	386	±	105	6	533	±	136
	10^6	6	653	±	161	5	914	±	195
	10^7	6	719	±	183	6	616	±	147
	10^9	6	594	±	156	6	251	±	101
	$10^6/10^7$	6	668	±	159	6	8494	±	4580
	$10^6/10^9$	6	600	±	151	6	1080	±	196
ob/ob	Uninoculated	3	153	±	21	3	51	±	4
	10^6	-	-	-	-	-	-	-	-
	10^7	3	264	±	28	3	387	±	20
	10^9	3	1593	±	60	3	2179	±	37
	$10^6/10^7$	-	-	-	-	-	-	-	-
	$10^6/10^9$	-	-	-	-	-	-	-	-
CBA	Uninoculated	-	-	-	-	-	-	-	-
	10^6	6	4820	±	428	6	5193	±	747
	10^7	6	5531	±	1056	6	4089	±	669
	10^9	6	7695	±	1499	6	8118	±	1099
	$10^6/10^7$	6	6250	±	654	6	6887	±	687
	$10^6/10^9$	6	5363	±	643	6	8027	±	1701
xid/Y	Uninoculated	6	63	±	26	6	56	±	6
	10^6	6	48	±	9	6	56	±	12
	10^7	6	64	±	11	6	71	±	9
	10^9	6	61	±	11	6	68	±	8
	$10^6/10^7$	6	72	±	11	6	63	±	8
	$10^6/10^9$	6	36	±	2	6	39	±	3

Blastogenic response with and without I/E: MLN with PHA stimulation

		Without I/E				With I/E			
		n	Mean	±	s.e.	n	Mean	±	s.e.
BALB/c	Uninoculated	12	19463	±	3219	12	22930	±	3175
	10^6	12	10865	±	1270	12	16393	±	2372
	10^7	12	9713	±	1930	12	15626	±	2732
	10^9	12	19650	±	5201	10	25812	±	4078
	$10^6/10^7$	9	15923	±	1136	9	14767	±	2849
	$10^6/10^9$	8	26555	±	5134	8	26098	±	4258
C57	Uninoculated	6	5046	±	2265	6	1793	±	662
	10^6	6	1776	±	304	6	1210	±	475
	10^7	6	927	±	203	6	2697	±	1360
	10^9	6	2460	±	695	6	2672	±	966
	$10^6/10^7$	6	1340	±	226	5	1479	±	703
	$10^6/10^9$	6	1965	±	521	6	1466	±	647
+/bg	Uninoculated	-	-	-	-	-	-	-	-
	10^6	3	395	±	102	3	463	±	32
	10^7	3	286	±	10	3	451	±	104
	10^9	3	266	±	37	3	396	±	162
	$10^6/10^7$	-	-	-	-	-	-	-	-
	$10^6/10^9$	-	-	-	-	-	-	-	-

bg/bg/

		Without I/E				With I/E			
		n	Mean	±	s.e.	n	Mean	±	s.e.
bg/bg	Uninoculated	3	22513	±	2182	3	25562	±	2212
	10^6	3	28482	±	2186	3	27918	±	2496
	10^7	3	32716	±	1302	3	35500	±	2681
	10^9	3	26285	±	2096	3	27	±	9
	$10^6/10^7$	3	31828	±	2089	3	38382	±	6938
	$10^6/10^9$	3	29832	±	1497	3	31429	±	885
ob/ob	Uninoculated	3	16044	±	875	3	17045	±	178
	10^6	-	-	-	-	-	-	-	-
	10^7	3	17432	±	1369	3	20690	±	1634
	10^9	3	22025	±	759	3	24147	±	842
	$10^6/10^7$	-	-	-	-	-	-	-	-
	$10^6/10^9$	-	-	-	-	-	-	-	-
CBA	Uninoculated	-	-	-	-	-	-	-	-
	10^6	3	40322	±	1452	3	45650	±	2797
	10^7	3	44864	±	4822	3	46714	±	3296
	10^9	3	57615	±	5496	3	59814	±	3454
	$10^6/10^7$	3	54887	±	4277	3	55178	±	6543
	$10^6/10^9$	3	53152	±	2042	3	49050	±	991
xid/Y	Uninoculated	3	303	±	94	3	884	±	291
	10^6	3	1307	±	215	3	3555	±	1318
	10^7	3	1318	±	355	3	2367	±	929
	10^9	3	847	±	393	3	1711	±	677
	$10^6/10^7$	3	1669	±	1126	3	1526	±	547
	$10^6/10^9$	3	612	±	300	3	1422	±	410

Blastogenic response with and without I/E: MLN with A. viscosus stimulation

		Without I/E				With I/E			
		n	Mean	±	s.e.	n	Mean	±	s.e.
BALB/c	Uninoculated	12	7075	±	1648	10	10953	±	2703
	10^6	11	7346	±	1477	11	12786	±	2022
	10^7	12	7126	±	1312	12	9825	±	1817
	10^9	10	7911	±	1545	12	10087	±	1806
	$10^6/10^7$	9	8134	±	2100	9	9225	±	2484
	$10^6/10^9$	9	7435	±	1919	9	8037	±	2136
C57	Uninoculated	6	3162	±	404	6	1292	±	351
	10^6	6	4190	±	1311	6	5058	±	2267
	10^7	6	8063	±	2589	6	24240	±	14997
	10^9	6	36302	±	15548	6	48609	±	23130
	$10^6/10^7$	6	37654	±	1716	6	31943	±	14377
	$10^6/10^9$	6	36486	±	15387	6	34449	±	15695
+/bg	Uninoculated	-	-	-	-	-	-	-	-
	10^6	3	50311	±	9565	3	41002	±	8552
	10^7	3	58132	±	13303	3	43020	±	4009
	10^9	2	5893	±	958	3	38870	±	1024
	$10^6/10^7$	-	-	-	-	-	-	-	-
	$10^6/10^9$	-	-	-	-	-	-	-	-
bg/bg/									

		Without I/E				With I/E			
		n	Mean	±	s.e.	n	Mean	±	s.e.
bg/bg	Uninoculated	3	1581	±	125	3	2756	±	190
	10^6	3	2401	±	207	3	4343	±	133
	10^7	3	2814	±	155	3	5991	±	172
	10^9	3	2383	±	488	3	4853	±	199
	$10^6/10^7$	3	3431	±	396	3	4985	±	415
	$10^6/10^9$	3	4000	±	137	3	5357	±	642
ob/ob	Uninoculated	3	4854	±	140	3	8145	±	936
	10^6	-	-	-	-	-	-	-	-
	10^7	3	2628	±	842	3	2934	±	436
	10^9	3	4462	±	425	3	9014	±	1129
	$10^6/10^7$	-	-	-	-	-	-	-	-
	$10^6/10^9$	-	-	-	-	-	-	-	-
CBA	Uninoculated	-	-	-	-	-	-	-	-
	10^6	3	16110	±	3030	3	23794	±	1722
	10^7	3	22059	±	2017	3	20866	±	4007
	10^9	3	29856	±	3841	3	33234	±	1113
	$10^6/10^7$	3	19721	±	1823	3	26188	±	6514
	$10^6/10^9$	3	21912	±	1524	3	21661	±	1500
xid/Y	Uninoculated	3	26	±	2728	3	88	±	23
	10^6	3	30	±	7	3	68	±	5
	10^7	3	39	±	7	3	60	±	15
	10^9	3	44	±	4	3	56	±	6
	$10^6/10^7$	3	63	±	4	3	54	±	2
	$10^6/10^9$	3	43	±	5	3	52	±	2

Blastogenic response with and without I/E: Spleen with zero stimulation

		Without I/E				With I/E			
		n	Mean	±	s.e.	n	Mean	±	s.e.
BALB/c	Uninoculated	19	1990	±	472	17	2014	±	414
	10^6	21	2544	±	490	17	2142	±	417
	10^7	19	2489	±	615	17	1587	±	289
	10^9	20	2078	±	534	18	1382	±	334
	$10^6/10^7$	16	1718	±	322	16	1617	±	215
	$10^6/10^9$	17	1097	±	146	18	1484	±	229
C57	Uninoculated	9	462	±	95	9	364	±	126
	10^6	9	416	±	102	9	727	±	295
	10^7	9	514	±	140	9	386	±	133
	10^9	8	365	±	65	9	180	±	50
	$10^6/10^7$	9	322	±	91	9	474	±	179
	$10^6/10^9$	9	331	±	82	9	369	±	131
+/bg	Uninoculated	6	9907	±	2249	6	9444	±	974
	10^6	6	7681	±	1715	6	13209	±	2046
	10^7	6	5517	±	2135	6	6137	±	2013
	10^9	6	11347	±	2499	6	9140	±	2401
	$10^6/10^7$	5	5428	±	1151	6	7216	±	2240
	$10^6/10^9$	6	7923	±	1630	6	7142	±	2634

bg/bg/

		Without I/E				With I/E			
		n	Mean	±	s.e.	n	Mean	±	s.e.
bg/bg	Uninoculated	6	549	±	87	6	642	±	168
	10^6	6	609	±	154	6	797	±	203
	10^7	6	809	±	167	6	881	±	268
	10^9	6	701	±	51	6	771	±	246
	$10^6/10^7$	6	1352	±	433	6	1548	±	515
	$10^6/10^9$	6	783	±	131	6	813	±	283
ob/ob	Uninoculated	3	355	±	16	3	105	±	10
	10^6	-	-	-	-	-	-	-	-
	10^7	3	1734	±	162	3	1706	±	196
	10^9	3	1709	±	97	3	2784	±	632
	$10^6/10^7$	-	-	-	-	-	-	-	-
	$10^6/10^9$	-	-	-	-	-	-	-	-
CBA	Uninoculated	6	9504	±	748	6	9849	±	1608
	10^6	6	13123	±	1057	6	13824	±	2203
	10^7	6	5360	±	1029	6	6957	±	1061
	10^9	6	16833	±	1324	6	5819	±	1500
	$10^6/10^7$	6	9551	±	740	6	6901	±	1076
	$10^6/10^9$	6	9411	±	1149	5	9266	±	1217
xid/Y	Uninoculated	6	69	±	7	6	63	±	8
	10^6	5	63	±	10	6	73	±	13
	10^7	6	51	±	12	6	60	±	12
	10^9	6	47	±	6	6	55	±	9
	$10^6/10^7$	6	70	±	8	6	54	±	9
	$10^6/10^9$	6	21	±	21	6	55	±	12

Blastogenic response with and without I/E: Spleen with PHA stimulation

		Without I/E				With I/E			
		n	Mean	±	s.e.	n	Mean	±	s.e.
BALB/c	Uninoculated	11	6725	±	1724	9	12444	±	4221
	10^6	12	10351	±	2444	8	15363	±	4060
	10^7	12	11395	±	3497	9	13419	±	4973
	10^9	11	5963	±	1798	9	9371	±	2836
	$10^6/10^7$	8	25293	±	6106	9	24419	±	3812
	$10^6/10^9$	9	35865	±	6281	9	27359	±	3422
C57	Uninoculated	6	826	±	254	6	1932	±	175
	10^6	6	2281	±	854	6	1953	±	392
	10^7	6	746	±	426	6	1604	±	368
	10^9	6	1794	±	513	6	2188	±	339
	$10^6/10^7$	6	638	±	134	6	1623	±	290
	$10^6/10^9$	6	1413	±	507	6	1549	±	282
+/bg	Uninoculated	3	16183	±	421	3	20548	±	963
	10^6	3	17019	±	1182	3	20663	±	601
	10^7	3	13208	±	1512	3	18914	±	3563
	10^9	3	21800	±	1358	3	13928	±	743
	$10^6/10^7$	3	12703	±	424	3	17512	±	399
	$10^6/10^9$	3	15193	±	1518	3	16628	±	650

bg/bg/

		Without I/E				With I/E			
		n	Mean	±	s.e.	n	Mean	±	s.e.
bg/bg	Uninoculated	3	8572	±	275	3	10745	±	1811
	10^6	3	7193	±	385	3	11224	±	1596
	10^7	3	12497	±	535	3	17706	±	2172
	10^9	3	4985	±	524	3	5005	±	1645
	$10^6/10^7$	3	11192	±	1175	3	13649	±	2255
	$10^6/10^9$	3	5306	±	152	3	6626	±	730
ob/ob	Uninoculated	3	6024	±	359	3	10175	±	351
	10^6	-	-	±	-	-	-	±	-
	10^7	3	3279	±	404	3	7568	±	276
	10^9	3	2933	±	241	3	5699	±	549
	$10^6/10^7$	-	-	-	-	-	-	-	-
	$10^6/10^9$	-	-	-	-	-	-	-	-
CBA	Uninoculated	3	33309	±	1718	3	37873	±	1079
	10^6	3	43334	±	3720	3	43396	±	4759
	10^7	3	28049	±	1404	3	29104	±	2408
	10^9	3	31826	±	1650	3	28597	±	5120
	$10^6/10^7$	3	30419	±	3101	3	33437	±	5054
	$10^6/10^9$	3	35778	±	3051	3	41353	±	2271
xid/Y	Uninoculated	3	499	±	163	3	858	±	269
	10^6	3	345	±	140	3	1550	±	591
	10^7	3	1161	±	372	3	1900	±	335
	10^9	3	2077	±	474	3	2196	±	426
	$10^6/10^7$	3	957	±	431	3	835	±	80
	$10^6/10^9$	3	684	±	157	3	1999	±	604

Blastogenic response with and without I/E: Spleen with A. viscosus stimulation

		Without I/E				With I/E			
		n	Mean	±	s.e.	n	Mean	±	s.e.
BALB/c	Uninoculated	9	7916	±	1847	9	12398	±	3210
	10^6	11	6952	±	1499	9	9541	±	2418
	10^7	11	6873	±	1431	9	8989	±	2211
	10^9	12	5668	±	1292	8	5000	±	2109
	$10^6/10^7$	9	7241	±	2034	9	7061	±	1867
	$10^6/10^9$	8	5311	±	1640	8	7986	±	2020
C57	Uninoculated	6	2994	±	230	6	4250	±	416
	10^6	6	4058	±	135	6	6556	±	886
	10^7	6	2537	±	369	6	4518	±	1140
	10^9	6	3411	±	200	6	4164	±	933
	$10^6/10^7$	6	3223	±	297	6	5532	±	1752
	$10^6/10^9$	6	3813	±	321	6	4010	±	1171
+/bg	Uninoculated	3	55121	±	3539	3	47207	±	2577
	10^6	3	56124	±	5907	3	53252	±	13409
	10^7	3	52271	±	6393	3	55526	±	5201
	10^9	3	48854	±	7096	3	46900	±	9900
	$10^6/10^7$	3	50054	±	4284	3	51471	±	5457
	$10^6/10^9$	3	53464	±	2208	3	46446	±	3558

bg/bg/

		Without I/E				With I/E			
		n	Mean	±	s.e.	n	Mean	±	s.e.
bg/bg	Uninoculated	3	792	±	55	3	2174	±	331
	10^6	3	980	±	105	3	2220	±	142
	10^7	3	1035	±	201	3	3576	±	333
	10^9	3	1081	±	144	3	1628	±	183
	$10^6/10^7$	3	1002	±	93	3	2596	±	139
	$10^6/10^9$	3	1332	±	125	3	2242	±	248
ob/ob	Uninoculated	3	1885	±	410	3	4448	±	568
	10^6	-	-	-	-	-	-	-	-
	10^7	3	1476	±	117	3	3614	±	746
	10^9	3	1854	±	603	3	179028	±	177174
	$10^6/10^7$	-	-	-	-	-	-	-	-
	$10^6/10^9$	-	-	-	-	-	-	-	-
CBA	Uninoculated	3	26075	±	3717	3	27986	±	2089
	10^6	3	19324	±	4128	3	24378	±	5512
	10^7	3	10786	±	888	3	15565	±	2070
	10^9	3	11403	±	1775	3	14533	±	3502
	$10^6/10^7$	3	11689	±	694	3	20053	±	2934
	$10^6/10^9$	3	18683	±	4225	3	21187	±	5555
xid/Y	Uninoculated	3	29	±	5	3	62	±	13
	10^6	3	49	±	10	3	58	±	10
	10^7	3	105	±	59	3	57	±	7
	10^9	3	53	±	2	3	99	±	25
	$10^6/10^7$	3	42	±	4	3	66	±	9
	$10^6/10^9$	3	38	±	3	3	58	±	10

C H A P T E R 8

IMMUNOGLOBULINS

8.1 I N T R O D U C T I O N

Periodontally diseased tissue contains higher levels of immunoglobulins than healthy tissue (Brandtzaeg, 1972; Clagett & Page, 1978). Much interest has therefore been centred on both their source and specificity.

8.1.1 SOURCE

It has been shown by many investigators that periodontitis patients are frequently seropositive for certain microorganisms in their gingival plaque (Mergenhagen et al, 1965; Genco et al, 1974; Listgarten et al, 1981; Doty et al, 1982; Ebersole et al, 1984; Tew et al, 1985b). The early assumption was that antibodies arrived in the gingival crevice as a serum transudate. However, more recent work (Tew et al, 1985a; Tynelius-Bratthall & Ellen, 1985) has demonstrated that certain specific immunoglobulins such as anti-Bacteroides gingivalis, anti-Actinobacillus actinomycetemcomitans and anti-A. viscosus are found at higher concentrations in gingival crevicular fluid than in serum, implying at least some local production. Local production is likely to be related to the observation that B-cells are the dominant infiltrate component in periodontal disease (Mackler et al, 1978a & b). It therefore seems reasonable to suppose that plaque-induced B-cell to plasma cell transformation occurs with subsequent local antibody production. However, the relative contributions of circulating and locally produced antibody to the immunoglobulin found in crevicular fluid are not known.

8.1.2 SPECIFICITY

While recent work has demonstrated that high levels of

specific antibody may be produced in response to periodontopathogens such as Actinobacillus actinomycetemcomitans and Bacteroides gingivalis (Tew et al, 1985b; Tynelius-Bratthall & Ellen, 1985), earlier work showed crevicular fluid antibodies to be largely non-specific (Brandtzaeg & Tolo, 1977; Clagett & Page, 1978). These authors thought that this bulk non-specificity was a result of the innumerable antigenic determinants in plaque. Also, if significant quantities of specific immunoglobulin were present, immune complex deposition would be anticipated. However, Berglund (1971) found only small amounts of immune complexes and Clagett and Page (1978) were unable to demonstrate any.

Various other studies have a bearing on lack of specificity. Banck & Forsgren (1978) found that 18 of 30 bacterial species tested induced mitogenesis in human B-lymphocyte cultures and Miller et al (1978) and Kunori et al (1978) showed that lipopolysaccharide (LPS), a component of all Gram-negative bacteria, has a potent mitogenic effect on the B-lymphocytes of human peripheral blood. Furthermore, Smith et al (1980), using the plaque-forming cell assay, found that human B-lymphocytes activated in vitro with preparations of suspected periodontopathogens produced antibody to unrelated antigens, in this case sheep red blood cells. Similarly, Mangan and Lopatin (1981) demonstrated that human lymphoid cells activated with a soluble fraction from Actinomyces viscosus, produced antibody unrelated to the activating substance. Thus, it appears that activation of B-cells, either within the gingival tissue or in vitro, with gingival crevicular debris or plaque bacteria, results in the production of immunoglobulins with a broad spectrum of

specificities (polyclonal activation). Such findings are not exclusive to humans, as Actinomyces viscosus, Actinomyces naeslundii, Actinomyces israelii, Bacteroides melaninogenicus, Fusobacterium, Actinobacillus actinomycetemcomitans and Capnocytophaga all evoke a strong mitogenic response in murine B-cells (Burckhardt et al, 1977; Engel et al, 1977; Diggens & Clagett, 1979; Smith et al, 1980; Carpenter et al, 1981; Chen et al, 1981; Mangan & Lopatin, 1981).

8.1.3 IMMUNOGLOBULIN LEVELS IN RESPONSE TO TREATMENT

Only recently have antibody levels to specific oral plaque bacteria been studied before and after periodontal treatment in man. Naito et al (1985) studied previously untreated periodontitis patients and examined the serum levels of IgG antibodies to seven different bacterial species, not including A. viscosus. These authors found that after treatment the response to Bacteroides gingivalis increased, that to Eikenella corrodens decreased while the responses to the others did not change. Tynelius-Brathall and Ellen (1985) studied the crevicular and salivary anti-A. viscosus and anti-A. naeslundii levels after treatment of gingivitis. They found tendencies for crevicular IgG and salivary IgG and IgA anti-A. viscosus levels to rise after treatment. Salivary IgG and IgA anti-A. naeslundii levels were barely detectable both before and after treatment. The explanation given for the anti-A. viscosus increase was that, in diseased tissues, antibodies would be consumed by interaction with antigens in excess, thus lowering the local antibody concentration. This is consistent with the findings of Doty et al

(1982) who reported higher serum antibody titres to certain periodontopathic bacteria in healing controls than in diseased subjects. On the other hand, total salivary IgA has been shown to be higher in subjects with periodontitis than those with healthy gingivae (Brandtzaeg, 1965; Shillitoe & Lehner, 1972; Lindstrom & Folke, 1973), while salivary IgG was found by Basu et al (1976) to decrease following periodontal therapy. Thus, such evidence as exists at present appears to be contradictory.

The data accumulated over the past decade, show that chronic human periodontitis is a predominantly B-lymphocyte/plasma cell lesion and that, except in the development of the early lesion, cell mediated hypersensitivity is not likely to be important (Page & Schroeder, 1982). This is not to say that T-cells are unimportant; they may play an essential role in regulating B-cells and plasma cells through their suppressor and helper activities. It is generally accepted that microbial substances from the gingival crevice and, in particular, the pocket, have access to the gingival tissues where they interact with lymphoid cells, causing B-lymphocyte activation and differentiation of large numbers of antibody-producing plasma cells. Most, if not all the microorganisms implicated as periodontal pathogens, are potent polyclonal activators of human B-lymphocytes (Page & Schroeder, 1982) so that antibody produced locally in the diseased gingival tissue is of mixed specificity. Consequently, immune complex formation either does not occur, or occurs to only a minor extent. On the other hand, as mitogens are more potent activators of lymphocytes than antigens and, since cells activated by mitogens

produce lymphokines (as do cells activated specifically by antigens), a high level of lymphokine production in diseased gingival tissues can be expected (Page & Schroeder, 1982).

The significance of such a potent local antibody response to B. gingivalis and A. actinomycetemcomitans (Tew et al, 1985b; Tynelius-Bratthall & Ellen, 1985) is not clear, but may relate to a basic pathogenic mechanism such as ability to invade the gingival lesion. The selectivity of the local response to these organisms is consistent with the hypothesis that these two species are important pathogens in early onset perdiodontitis. This is also supported by the case report of Haffajee et al (1984) which indicated that most sites with elevated levels of anti-A. actinomycetemcomitans antibodies suffered periodontal damage after the detection of the local antibody response. The question of whether these two organisms are unique in their ability to induce such potent local antibody responses in patients with juvenile periodontitis and severe generalised periodontitis remains open. Further study is indicated to determine whether advanced local production of antibody, detectable in gingival crevicular fluid, is related to active disease progression.

8.1.4 THE PRESENT STUDY

Immunoglobulin studies were designed to examine both the non-specific and specific anti-A. viscosus extract serum immunoglobulin levels in classes IgG, IgA and IgM in an attempt to determine the role of the humoral immune system in the induction of disease in the A. viscosus mouse model.

8.2 MATERIALS AND METHODS

All immunoglobulin studies were performed using an indirect Enzyme-Linked Immunosorbent Assay (ELISA) technique on serum from all genotypes except bg/bg and +/xid for the non-specific assays and all except +/xid for the specific assays.

8.2.1 THE ELISA TECHNIQUE

This technique, as described by Voller et al (1980), involves the placement of known antigen at known concentration on the bottom of the wells of disposable polystyrene plates. The test serum, whose levels of antibody to the known antigen are to be determined, is added to the plates. Subsequently, enzyme-conjugated antiglobulin and finally enzyme substrate are added resulting in the development of a colour change, the depth of which, as read by a colorimetric detector, is directly related to the concentration of antibody present in the test serum.

All ELISA assays were performed in triplicate, with a standard mouse serum used for all experiments coming from 20 BALB/c mice, each of which had been inoculated subcutaneously with 10^7 cfu A. viscosus in 50 µl of supplemented PPY. These animals were bled immediately postmortem three weeks later and the resultant serum was pooled, aliquoted into 0.25 ml portions and stored at -40°C till required. This standard serum was used at a 1 in 1000 dilution for the non-specific ELISAs and undiluted for the specific anti-A. viscosus assays. Each test serum was a pooled sample from all mice of the same genotype and inoculum group.

8.2.2 NON-SPECIFIC IMMUNOGLOBULIN

Rabbit anti-mouse antiserum IgG, IgA or IgM (Sigma Chemical Company Ltd, Poole, UK) was diluted to the appropriate working concentration in coating buffer (see Appendix) and 150 µl aliquots plated into individual wells in polystyrene, disposable, flat-bottomed, 96-well microtitration plates (Flow Labs, Rickmansworth, UK). IgG was used at a 1 in 500 dilution while both IgA and IgM were used at a 1 in 100 dilution, with separate plates for each Ig class. The plates were then stored for 18 h at 4°C to allow them to become sensitised by passive adsorption. All plates were then washed three times in a phosphate buffered saline-tween 20 mixture (PBS-tween) (see Appendix) using a mini-microwash system (Flow Labs, Rickmansworth, UK) and then tapped dry. To each well was then added 100 µl of a 1% mixture of newborn calf serum (NCS) in PBS-tween. To the first well of the first row of each plate was added 50 µl of 1 in 1000 diluted standard serum in 1% NCS-PBS-tween. The second row had no serum added and acted as a negative control. The first wells of all the remaining rows had 50 µl of the undiluted test serum added, and serial dilutions of all sera, both test and standard, were performed along the rows. All plates were then incubated at room temperature for 1.5 h after which all were washed three times in PBS-tween and tapped dry. To each well was then added 100 µl of the appropriate Ig class alkaline-phosphate-conjugated goat anti-mouse serum, diluted 1 in 1000 in 1% NCS-PBS-tween. The plates were incubated at room temperature for 1 h and again washed three times in PBS-tween and tapped dry. Each well then had 100 µl of phosphate substrate solution placed

in it (see Appendix) and, once a yellow colour had developed, all plates were read in the Multiskan Detector (Flow Labs, Rickmansworth, UK). This machine could be programmed to regard the second row of each plate as a zero blank, thus allowing for any slight yellowing due to non-specific change of the substrate.

The actual concentrations of total IgG, IgA and IgM in the standard serum were then measured by performing the same ELISA technique on the standard serum and a mouse immunoglobulin reference serum (Miles Scientific, Slough, UK). Thus, immunoglobulin concentrations could be expressed in mg ml^{-1} serum.

8.2.3 SPECIFIC ANTI-A. VISCOSUS IMMUNOGLOBULIN

The procedure used for measuring the concentrations of specific anti-A. viscosus immunoglublin was as detailed for the non-specific assays, except that undiluted standard serum was used and that the plates were sensitised with 100 µl of the EDTA A. viscosus extract per well instead of the rabbit anti-mouse antiserum. However, it was necessary to test which concentration of the A. viscosus extract would be appropriate.

The protein content of each A. viscosus extract was estimated by the Lowry method and concentrations of 1, 10 and 100 µg ml^{-1} were prepared and a standard ELISA using these was performed as above. The optimum concentration in terms of colour change of 50 µg ml^{-1} was then chosen and used for all the specific ELISAs.

8.3 R E S U L T S

8.3.1 SOURCES OF VARIATION

Nested analysis of variance showed that for both non-specific (Table 8.1) and specific (Table 8.2) immunoglobulins of all three classes there was statistically significant variation between genotypes and between inoculum groups. For the specific immunoglobulins, where two pooled samples were studied, there was a significant difference between samples for IgG but not for IgA or IgM.

8.3.2 NON-SPECIFIC IMMUNOGLOBULIN AND INOCULUM SIZE

For the non-specific immunoglobulin assays (Table 8.3) there was a similar pattern of results for both IgG and IgM, with immunoglobulin concentration tending to increase with inoculum size in all genotypes except xid/Y. The only exception was for IgM in ob/ob mice where serum from the 10^9 cfu group showed a significantly lower immunoglobulin concentration than that from the 10^7 group. One way analysis of variance for each of these two immunoglobulin classes showed that, of the total of 27 possible comparisons between pairs of inoculum groups within genotypes (except xid/Y), 23 comparisons for IgG and 23 for IgM showed a statistically significant difference ($P < 0.05$). For xid/Y, no two inoculum groups were significantly different for either IgG or IgM.

By contrast, the IgA results showed a markedly positive relationship between immunoglobulin concentration and inoculum size for xid/Y with a similar though generally less clear-cut trend for the other genotypes except C57, where there was a

definite peak at 10^7 cfu. The only difference that failed to reach statistical significance for xid/Y was that between the 10^6 and 10^7 cfu groups, while 19 of the 27 possible comparisons between pairs of inoculum groups within the other genotypes showed a statistically significant difference ($P < 0.05$).

8.3.3 SPECIFIC IMMUNOGLOBULIN AND INOCULUM SIZE

For the specific anti-A. viscosus immunoglobulin assays (Table 8.4) there was a general tendency for immunoglobulin concentration to increase with inoculum size for both IgG and IgM in all genotypes, including xid/Y. The most marked exception was for IgM in ob/ob mice, where serum from the 10^9 cfu group showed a significantly lower specific immunoglobulin concentration, not only compared with serum from the 10^7 cfu group, as found for non-specific immunoglobulin, but also compared with that from uninoculated animals ($P < 0.05$). Also for IgM, there were no significant differences between inoculum groups for bg/bg. One way analysis of variance for each of these two immunoglobulin classes showed that of the 39 possible comparisons between pairs of inoculum groups within genotypes, 26 showed a statistically significant difference for IgG and 22 for IgM.

By contrast, the IgA concentrations showed a marked peak at 10^7 cfu for all genotypes except xid/Y. For each of the genotypes BALB/c, C57, +/bg, and ob/ob, the concentration of IgA in serum from the 10^7 cfu group was significantly greater than that in serum from all other inoculum groups ($P < 0.05$). For bg/bg, although a similar peak at 10^7 cfu was observed, the

differences between inoculum groups were not statistically significant. For the increase in IgA concentration with inoculum size in xid/Y mice, four of the six comparisons between inoculum groups showed a statistically significant difference ($P < 0.05$).

8.3.4 THE RATIO OF SPECIFIC/NON-SPECIFIC IMMUNOGLOBULIN

The ratio of specific/non-specific immunoglobulin concentration for each genotype and inoculum group is shown in Table 8.5. Specific immunoglobulin amounted to only 1-300 parts per million non-specific immunoglobulin.

Table 8.1 Nested analysis of variance for non-specific immunoglobulin concentrations in mg μl^{-1} pooled serum. The denominator for all F ratios is the between repeat measurements mean square.

Immunoglobulin	Source of variation	ss	DF	ms	F	Significance
IgG	Between genotypes	91902	5	18380.4	82.2	0.000
	Between inoculum groups within genotypes	202365	17	11903.8	53.2	0.000
	Between repeat measurements within inoculum groups	8726	39	223.8		
IgA	Between genotypes	146156	5	29231.3	398.8	0.000
	Between inoculum groups within genotypes	189454	17	11144.4	152.1	0.000
	Between repeat measurements within inoculum groups	2858	39	73.3		
IgM	Between genotypes	836	5	167.1	210.5	0.000
	Between inoculum groups within genotypes	1126	17	66.3	83.4	0.000
	Between repeat measurements within inoculum groups	31	39	0.8		

Table 8.2 Nested analysis of variance for specific anti-*A. viscosus* immunoglobulin concentration in mg ml^{-1} pooled serum for IgG and mg 100 ml^{-1} pooled serum for IgA and IgM. The denominator for all F ratios is the between repeat measurements mean square.

Immunoglobulin	Source of variation	ss	DF	ms	F	Significance
IgG	Between genotypes	4539	6	756.5	60.6	0.000
	Between inoculum groups within genotypes	9332	20	466.6	37.4	0.000
	Between samples within inoculum groups	1021	19	53.7	4.3	0.000
	Between repeat measurements within samples	10734	86	12.5		
IgA	Between genotypes	178183	6	29697.2	13.1	0.000
	Between inoculum groups within genotypes	774013	20	38700.7	17.0	0.000
	Between samples within inoculum groups	21800	19	1147.4	0.5	0.953
	Between repeat measurements within samples	172973	76	2276.0		

Table 8.2 (Continued)

Immunoglobulin	Source of variation	ss	DF	ms	F	Significance
IgM	Between genotypes	3329	6	554.8	51.5	0.000
	Between inoculum groups within genotypes	2753	20	137.7	12.8	0.000
	Between samples within inoculum groups	236	19	12.4	1.5	0.317
	Between repeat measurements within samples	926	86	10.8		

Table 8.3 Non-specific immunoglobulin concentration by genotype and inoculum group in mg μl^{-1} pooled serum. n = number of repeat measurements made on pooled sample.

		IgG				IgA				IgM			
INOCULUM:		0	10^6	10^7	10^9	0	10^6	10^7	10^9	0	10^6	10^7	10^9
GENCTYPE													
BALB/c	n	2	2	2	2	2	2	2	2	2	2	2	2
	Mean	13.13	50.13	113.38	220.80	176.67	189.92	212.00	207.59	1.90	3.23	10.64	11.78
	s.e.	1.94	4.77	5.97	5.97	0	4.42	8.83	4.42	0.38	0.57	0.76	1.14
C57	n	3	3	3	3	3	3	3	3	3	3	3	3
	Mean	66.04	99.46	167.09	210.85	14.43	38.87	69.19	24.44	1.68	7.28	10.92	16.78
	s.e.	3.47	24.20	6.89	17.34	0.29	4.67	2.55	1.28	0.11	0.84	0.48	1.38
+/bg	n	3	3	3	3	3	3	3	3	3	3	3	3
	Mean	143.22	175.05	189.37	216.42	35.33	31.80	70.67	125.44	7.85	8.49	16.97	17.23
	s.e.	5.51	5.74	4.21	6.94	2.04	4.08	7.36	4.67	0.25	0.25	0.67	0.55
ob/ob	n	2		2	2	2		2	2	2		2	2
	Mean	145.61		179.02	226.76	39.75		61.83	61.83	5.51		18.62	11.78
	s.e.	2.39		11.94	11.94	0.88		1.77	5.30	0.19		0.38	0.38

Table 8.3 (Continued)

		IgG				IgA				IgM			
INOCULUM:		0	10^6	10^7	10^9	0	10^6	10^7	10^9	0	10^6	10^7	10^9
GENOTYPE													
CBA	n	3	3	3	3	3	3	3	3	3	3	3	3
	Mean	41.38	197.32	219.60	290.42	21.38	47.11	46.52	288.56	2.05	3.67	7.35	12.67
	s.e.	2.10	3.47	6.32	10.53	0.87	1.56	3.28	15.58	0.12	0.13	0.25	0.63
xid/Y	n	3	3	3	3	3	3	3	3	3	3	3	3
	Mean	95.48	93.89	93.89	101.84	11.54	48.29	57.71	131.91	1.93	1.99	1.93	2.25
	s.e.	0	1.59	1.59	8.86	1.31	3.12	3.12	5.13	0.11	0.14	0.03	0.11

Table 8.4 Specific anti-A. viscosus immunoglobulin concentration by genotype and inoculum group in mg ml^{-1} pooled serum for IgG and mg 100 ml^{-1} pooled serum for IgA and IgM. n = total number of repeat measurements made on either one or two pooled samples.

		IgG				IgA				IgM			
INOCULUM:		0	10^6	10^7	10^9	0	10^6	10^7	10^9	0	10^6	10^7	10^9
GENOTYPE													
BALB/c	n	6	6	6	6	5	6	5	6	6	6	6	6
	Mean	4.10	9.79	8.55	26.14	30.18	77.56	283.59	47.88	4.08	4.92	5.56	7.69
	s.e.	1.09	1.06	1.39	3.69	3.81	14.42	37.00	7.102	0.69	0.82	0.69	0.85
C57	n	6	6	6	6	6	6	6	6	6	6	6	6
	Mean	6.85	6.99	16.50	24.76	39.63	53.47	302.10	57.53	10.83	9.42	13.41	27.97
	s.e.	2.16	0.55	3.31	4.84	7.36	11.67	68.07	6.66	1.15	0.96	1.75	3.76
+/bg	n	6	6	6	6	5	5	5	5	6	6	6	6
	Mean	2.78	3.74	7.34	11.84	12.37	24.73	73.85	49.11	3.97	6.92	12.10	15.00
	s.e.	0.54	0.17	0.55	0.97	3.49	4.71	6.94	4.98	0.43	0.44	0.91	0.87
bg/bg	n	3	3	3	3	2	2	2	2	3	3	3	3
	Mean	4.46	5.17	9.87	14.32	18.11	12.19	44.17	27.38	5.45	7.22	6.90	9.31
	s.e	1.86	1.17	3.54	2.30	17.22	3.36	40.63	0.88	2.28	1.37	1.48	1.98

Table 8.4 (Continued)

		IgG				IgA				IgM			
INOCULUM:		0	10^6	10^7	10^9	0	10^6	10^7	10^9	0	10^6	10^7	10^9
GENOTYPE													
ob/ob	n	4		4	4	4		4	4	4		4	4
	Mean	13.49		30.13	40.28	71.55		245.57	49.25	6.95		7.93	3.90
	s.e.	0.60		1.78	0.33	4.65		7.97	6.31	0.24		0.59	0.36
CBA	n	6	6	6	6	6	6	6	6	6	6	6	6
	Mean	2.27	14.08	19.02	32.94	22.08	60.21	229.67	30.33	11.02	17.10	15.36	27.23
	s.e.	0.12	0.88	1.03	0.69	2.18	4.08	11.85	5.41	1.72	1.04	1.69	2.27
xid/Y	n	3	3	3	3	3	3	3	3	3	3	3	3
	Mean	1.27	3.90	4.61	28.64	2.59	10.01	11.78	13.54	1.84	2.66	3.10	6.97
	s.e.	0.08	0.21	0.21	3.65	0.51	0.59	1.56	0.59	0.23	0.11	0.17	0.34

Table 8.5 Ratio of specific/non-specific immunoglobulin concentration for each genotype and inoculum group (x 10^6).

	IgG				IgA				IgM			
INOCULUM:	0	10^6	10^7	10^9	0	10^6	10^7	10^9	0	10^6	10^7	10^9
GENOTYPE												
BALB/c	312.3	195.3	75.4	118.4	1.7	4.1	13.4	2.3	21.5	15.2	5.2	6.5
C57	103.7	70.3	98.7	117.4	27.5	13.8	43.7	23.5	64.5	12.9	12.3	16.7
+/bg	19.4	21.4	38.8	54.7	3.5	7.8	10.4	3.9	5.1	8.2	7.1	8.7
ob/ob	92.6		168.3	177.6	18.0		39.7	8.0	12.6		4.3	3.3
CBA	54.9	71.4	86.6	113.4	10.3	12.8	49.4	1.1	53.8	46.6	20.9	21.5
xid/Y	13.3	41.5	49.1	281.2	2.2	2.1	2.0	1.0	9.5	13.4	16.1	31.0

8.4 D I S C U S S I O N

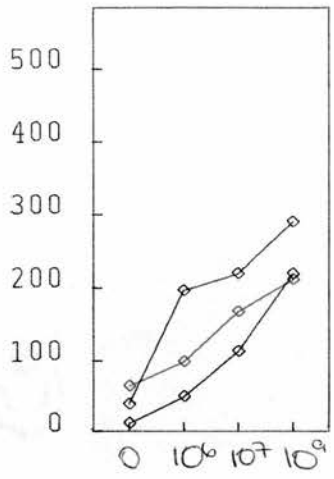
The relevance of the results lies in the response, in terms of immunoglobulin production, to increasing numbers of organisms inoculated, and in the differences of response between genotypes, between non-specific and specific anti-A. viscosus immunoglobulins and between immunoglobulin classes. The patterns of response observed are summarised in Table 8.6 and are represented diagrammatically in Figures 8.1-8.8.

Table 8.6 Summary of the relationship between immunoglobulin concentration and inoculum size for the different genotypes.

		Non-specific	Specific
IgG	1.	BALB/c, C57, +/bg, ob/ob, CBA	1. All genotypes except +/xid
	2.		2.
	3.	xid/Y	3.
	4.	bg/bg, +/xid	4. +/xid
IgA	1.	BALB/c, +/bg, ob/ob, CBA, xid/Y	1. xid/Y
	2.	C57	2. BALB/c, C57, +/bg, ob/ob, CBA
	3.		3. bg/bg
	4.	bg/bg, +/xid	4. +/xid
IgM	1.	BALB/c, C57, +/bg, CBA	1. BALB/c, C57, +/bg, CBA, xid/Y
	2.	ob/ob	2. ob/ob
	3.	xid/Y	3. bg/bg
	4.	bg/bg, +/xid	4. +/xid

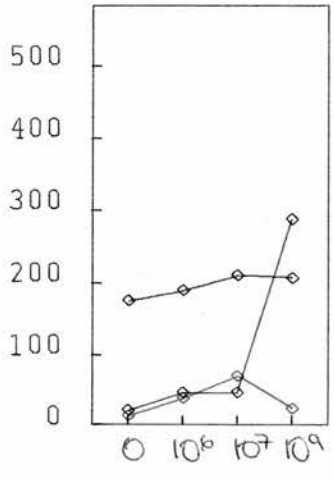
1. = Increase in immunoglobulin concentration with inoculum size
2. = Statistically significant peak at 10^7 cfu
3. = No significant difference between inoculum groups
4. = No data

IgG



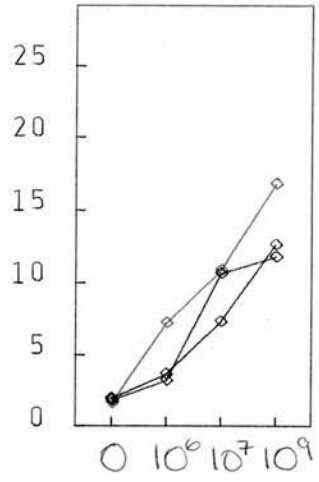
Inoculum:

IgA



Inoculum:

IgM



Inoculum:

Fig 8.1 Mean non-specific immunoglobulin concentration by inoculum group in mg μl^{-1} pooled serum. BALB/c (green), C57 (red), CBA (black).

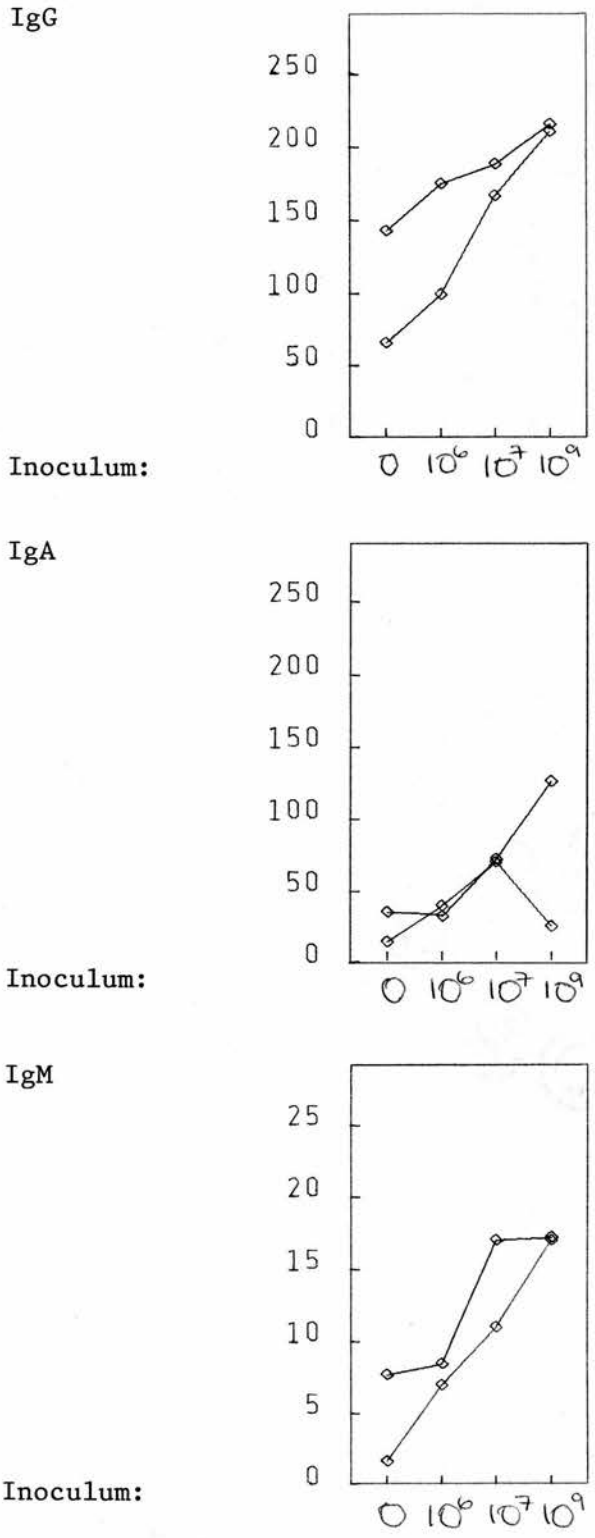
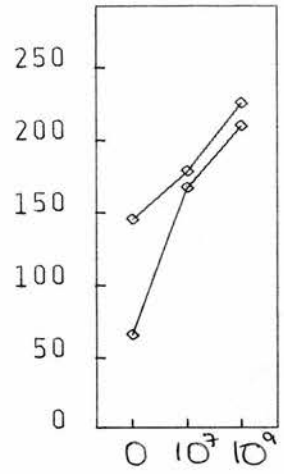


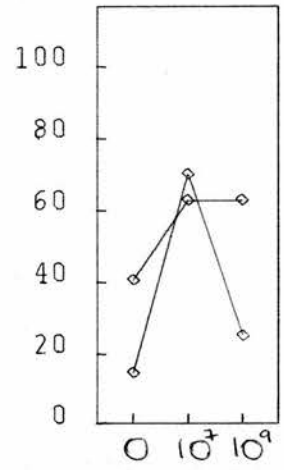
Fig 8.2 Mean non-specific immunoglobulin concentration by inoculum group in mg μl^{-1} pooled serum. C57 (black), +/bg (green).

IgG



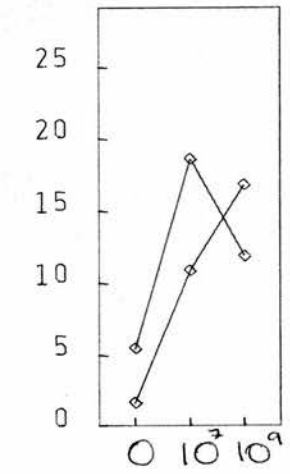
Inoculum:

IgA



Inoculum;

IgM



Inoculum:

Fig 8.3 Mean non-specific immunoglobulin concentration by inoculum group in mg μl^{-1} pooled serum. C57 (black), ob/ob (red).

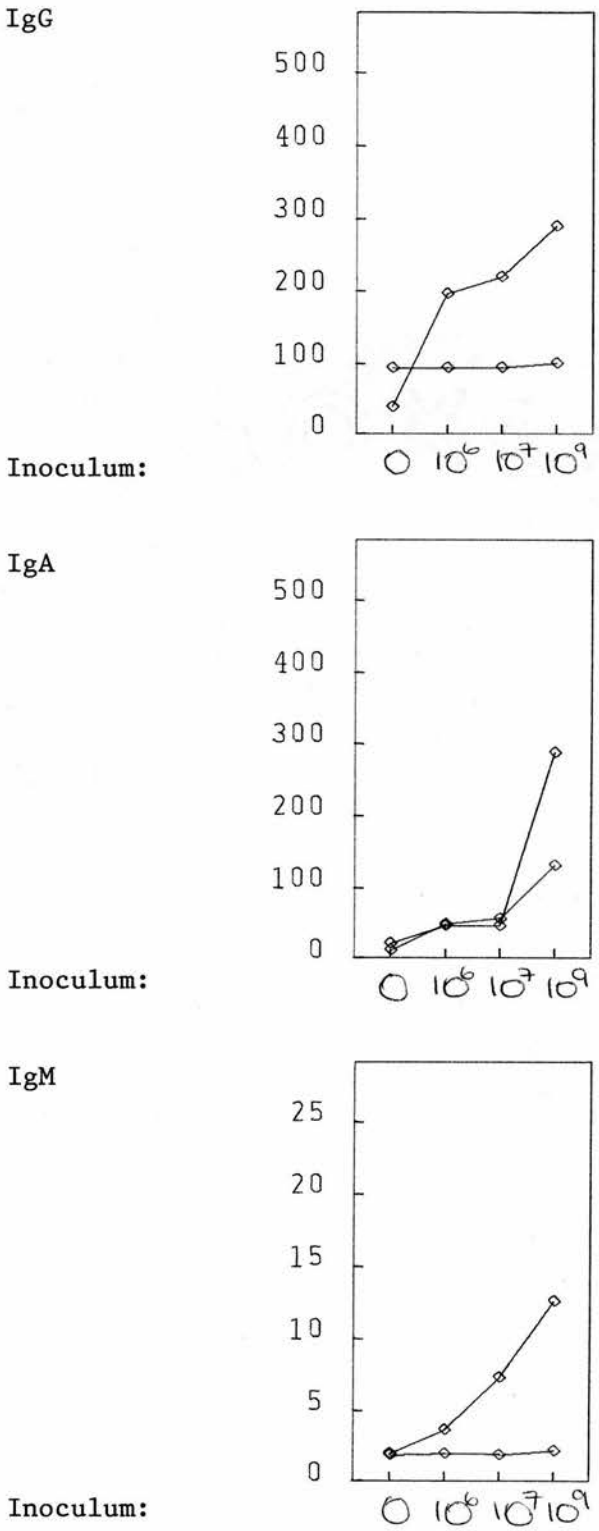
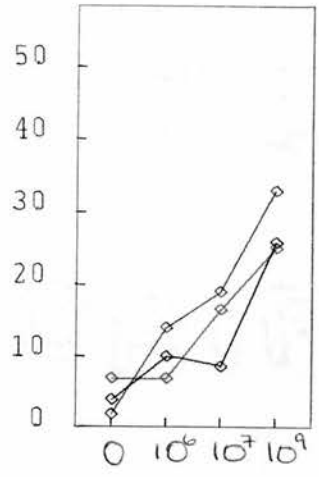
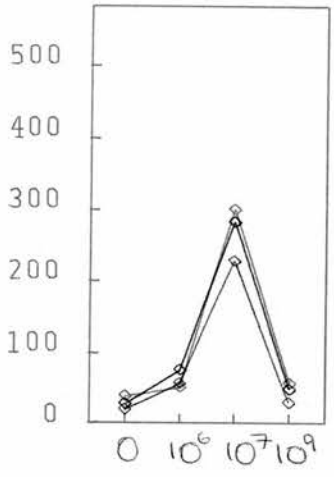


Fig 8.4 Mean non-specific immunoglobulin concentration by genotype and inoculum group in mg μl^{-1} pooled serum.
CBA (black), xid/Y (red).

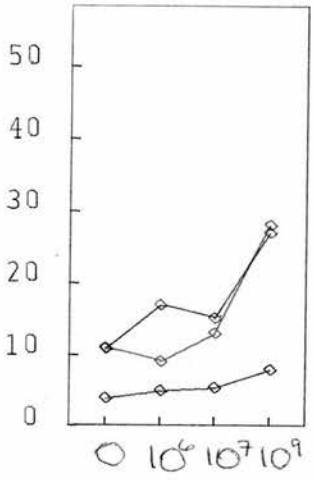
IgG



IgA



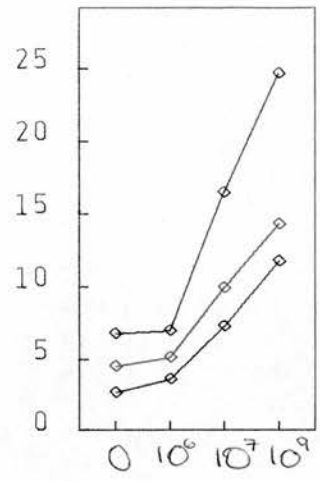
IgM



Inoculum:

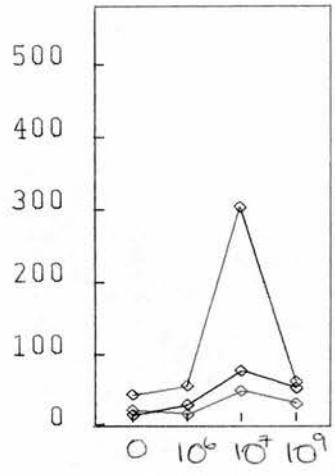
Fig 8.5 Mean specific anti-A. viscosus immunogloublin concentration by inoculum group in mg ml^{-1} for IgG and mg 100 ml^{-1} for IgA and IgM pooled serum. BALB/c (green), C57 (red), CBA (black).

IgG



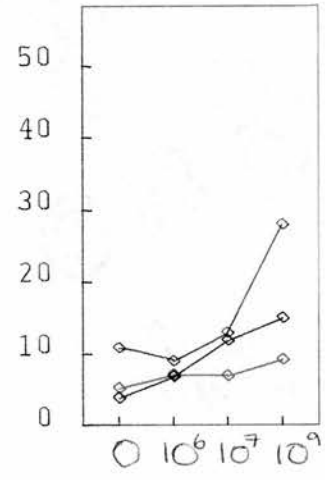
Inoculum:

IgA



Inoculum:

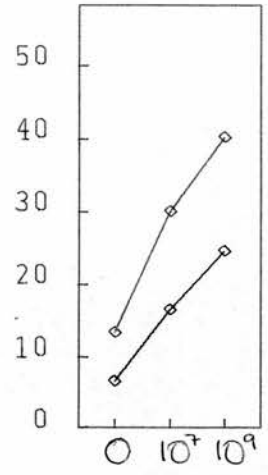
IgM



Inoculum:

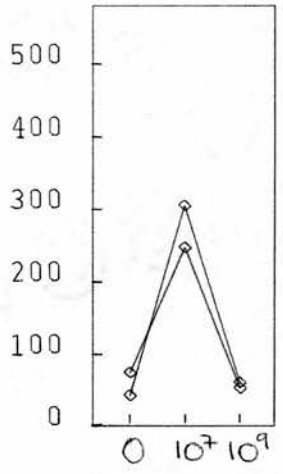
Fig 8.6 Mean specific anti-A. viscosus immunoglobulin concentration by inoculum group in mg ml^{-1} for IgG and mg 100 ml^{-1} for IgA and IgM pooled serum.
C57 (black), +/bg (green), bg/bg (red).

IgG



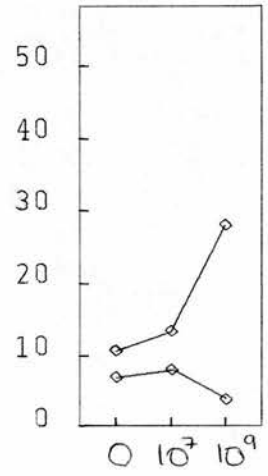
Inoculum:

IgA



Inoculum:

IgM



Inoculum:

Fig 8.7 Mean specific anti-A. viscosus immunoglobulin concentration by inoculum group in mg ml^{-1} for IgG and mg 100 ml^{-1} for IgA and IgM pooled serum.
C57 (black), ob/ob (red).

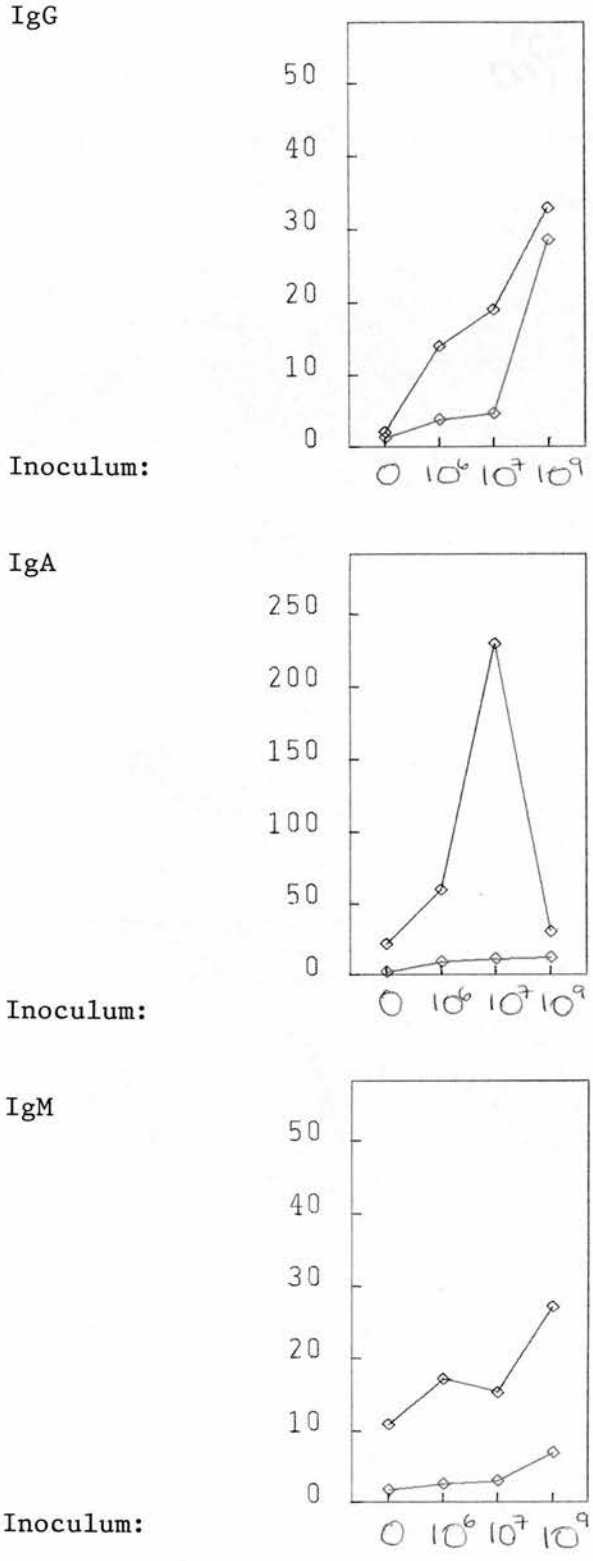


Fig 8.8 Mean specific anti-A. viscosus immunoglobulin concentration by inoculum group in mg ml^{-1} for IgG and mg 100 ml^{-1} for IgA and IgM pooled serum. CBA (black), xid/Y (red).

8.4.1 DIFFERENCES BETWEEN GENOTYPES

Table 8.6 shows that in classes IgG and IgM of both non-specific and specific anti-A. viscosus ELISAs, there was a general tendency for Ig concentration to increase with increasing inoculum size, indicating that the host's immune system was responding to increasing exposure to A. viscosus. Exceptions to this pattern were found for genotypes ob/ob in class IgM of both non-specific and specific ELISAs, xid/Y in classes IgG and IgM in the non-specific ELISAs only and bg/bg in classes IgA and IgM in the specific ELISA tests.

As far as ob/ob mice are concerned, this may have been associated with defects of cell-mediated immunity thought to occur in obese mice (Meade et al, 1979; Clark et al, 1981), although it has been postulated that these defects may be the result of the metabolically abnormal tissue environment rather than intrinsic defects of the immune system (Meade et al, 1979; Chandra, 1980). If cellular immune function in these animals is defective, however, this could profoundly affect the B-cells and thus Ig levels through alteration of T-cell help or suppressive activities.

The xid defect is characterised by the inability of affected mice to mount an antibody response to various helper T-cell independent antigens (Amsbaugh et al, 1972,1974; Scher et al, 1973; Press, 1981; Scher, 1982b). It may be that A. viscosus antigens fall into this category. Metcalf et al (1980) found xid mice to be extremely susceptible to tolerance, which could perhaps explain the pattern of immunoglobulin concentrations in classes

IgG and IgM of the non-specific ELISAs. Perhaps the most likely explanation, however, is the decreased magnitude of response of the secretory immune system of these mutants as described by Kiyono et al (1983).

The serum of bg/bg mice showed no significant difference between inoculum groups in respect of the specific anti-A. viscosus tests in classes IgA and IgM. Some other defect, such as that of NK function, as detailed in Chapter 2, may be responsible.

8.4.2 DIFFERENCE BETWEEN NON-SPECIFIC AND SPECIFIC IMMUNOGLOBULINS

It is noticeable that Table 8.5 records a non-zero ratio of specific/non-specific Ig for uninoculated animals, even though the recovery experiments (Section 5.3) showed that these animals did not carry A. viscosus in their mouths. It is possible that this anomalous finding was caused by cross-reaction of certain A. viscosus antigens with those of commensal organisms. Table 8.5 also shows that, for both IgG and IgM, there was no consistent pattern of ratios, indicating comparable responses to inoculum size by both specific and non-specific Igs. For IgA, however, the ratio peaked at 10^7 cfu in all genotypes except xid/Y, reflecting the difference of response between specific and non-specific IgA, as summarised in Table 8.6. 0

8.4.3 DIFFERENCES BETWEEN IMMUNOGLOBULIN CLASSES

In class IgG, the variation between samples was significantly greater than that between repeat measurements within samples

(Table 8.2), which may imply that the assay technique was more sensitive for IgG, perhaps through greater specificity for the test sera of this class of antiserum compared with IgA and IgM.

The ratio of specific/nonspecific IgG was generally higher than for either IgA or IgM. This is consistent with the explanation that, in the course of normal eating and swallowing, there was continued organism ingestion in sufficient numbers to induce the equivalent of a secondary antibody response, characterised by a predominance of IgG (Amos, 1981).

8.4.4 THE IgA PEAK AT 10^7 CFU

The most important point to emerge from the Ig studies was the peak found in specific anti-A. viscosus levels associated with the 10^7 cfu inoculations which were, in turn, consistently associated with the greatest bone loss (Section 3.4).

The IgA peak at 10^7 cfu is consistent with high and low zone tolerance having been induced by oral inoculations of 10^9 and 10^6 cfu live A. viscosus respectively, as discussed in Section 3.4.3. The present findings are compatible with those of Andre (1975) who showed that the serum from intragastrically immunised mice contained an active toleragen which seemed to consist of immune complexes with IgA as the antibody. It had earlier been demonstrated that intragastric administration of antigens caused the appearance of circulating antibodies, often predominantly of the IgA class (Heremans, 1974). It is, therefore, logical to assume that orally inoculated antigen would induce a similar response, since much of it must be swallowed, and that comparable immune complexes might be formed.

Immune complexes (ICs) form as a result of the combination of antibodies with their evoking antigenic determinants. ICs formed in great antigen excess are small, do not fix complement and generally cannot initiate inflammatory processes. On the other hand, ICs formed at great antibody excess, although capable of activating complement, are large, insoluble, rapidly phagocytosed and thus of limited pathogenicity. The greatest pathological potential seems to lie between these two extremes when ICs are of intermediate size, soluble and large enough to fix complement (Theofilopoulos & Dixon, 1980). If the host's exposure to antigen is of limited duration, even though some complexes form, tissue injury and other clinical manifestations are transient, as in classical 'one shot serum sickness' (Wilson & Dixon, 1970, 1971). In contrast, if the antigen remains long in the circulation, or if there is a continuous supply of antigenic material, as with chronic infections or autoantigens, the potential exists for continuing IC formation. The closest approximation to such chronic IC disease in man is 'chronic' serum sickness (Wilson & Dixon, 1976). It is possible that inoculated mice in the current work, by virtue of their continued oral infection through oral colonisation, fall into this latter category.

When ICs arise, their reaction with complement leads to many complex molecular events. Among these are cleavage of C3, binding of C3b to the IC, release of C3a, and, on cleavage of C5, release of C5a. In acting on mast cells, C3a and C5a effect histamine release and thus increased capillary permeability. In acting on polymorphs, the C5a component chemotactically attracts leucocytes

to the site of IC deposition. There, leucocytes are trapped by IC-bound C3b, C3bi, C3d and C4b, which exhibit reactivity with the various complement receptors on the leucocytes. Subsequently, the accumulated leucocytes release hydrolases and cause tissue injury (Dixon, 1971; Cochrane & Dixon, 1978).

The gingivae have a good blood supply with a relatively high blood flow per unit mass of tissue and are thereby exposed to and can trap large quantities of ICs in their vascular walls. The damaging sequellae of IC presence, as detailed above, could thus lead to the local damage found in the experimental mice of the current work.

8.5 C O N C L U S I O N

It appears that, in the mouse model studied, the most important correlation between Ig concentration and induced disease occurred for the specific anti-A. viscosus IgA level. It is possible that IgA reacted with A. viscosus antigen to produce ICs which resulted in local damage. It is also possible that there may have been some form of tolerance associated with 10^6 cfu and 10^9 cfu inoculations. Although no supporting evidence of such tolerance was apparent from the serum studies, it could be that saliva studies would be more informative in relation to local Ig production. Fitzgerald et al (1981) found that serum antibody levels "did not increase significantly above baseline levels in any colonised animals". Thus, no comparison of the present results with previous work is possible.

C H A P T E R 9

IMMUNISATION

9.1 INTRODUCTION

In view of the consistency with which maximum bone loss was induced by oral inoculations of 10^7 cfu A. viscosus over all genotypes, it seemed appropriate to attempt immunisation of 10^7 cfu inoculated mice. Immunisation experiments were carried out only on BALB/c mice fed the special diet.

9.2 MATERIALS AND METHODS

All mice involved in immunisation procedures received oral inoculations of 10^7 cfu A. viscosus as described in Section 2.4. Measurement of bone loss, lymphocyte transformation and ELISA tests were carried out 12 weeks after oral inoculation as described in previous chapters.

Three immunisation procedures were attempted. For each, a group of ten BALB/c mice was used:

Group '9-7': 50 µl subcutaneous inoculation containing 10^9 cfu heat-killed A. viscosus on each of three successive days two weeks before oral inoculation.

Group '7-9': 50 µl subcutaneous inoculation containing 10^9 cfu heat-killed A. viscosus on each of three successive days two weeks after oral inoculation.

Group 'IV': 50 µl intravenous inoculation of 10^5 cfu live A. viscosus into the lateral tail vein on each of three successive days two weeks before oral inoculation.

For bone loss measurements two further groups were studied, a group of uninoculated and unimmunised mice, and a group of mice given oral inoculations of 10^7 cfu only. These groups were

investigated to provide confirmation of bone loss observed in the main experiment and baseline levels for the immunisation studies.

Heat killing was achieved by placing glass vials of A. viscosus culture in an 80°C water bath for 10 mins. The efficacy of this treatment was determined by plating samples of treated culture on SA plates in triplicate.

9.3 R E S U L T S

No heat-treated cultures of A. viscosus yielded growth indicating efficacy of the killing procedure.

Bone loss findings are summarised in Table 9.1. The three immunised groups, their uninoculated and 10^7 cfu controls, with corresponding uninoculated and 10^7 cfu groups from the main experiment, are included. The 7-9 and 9-7 groups were not significantly different from each other or the 10^7 cfu groups, showing that these immunisation procedures were ineffective. The IV group was not significantly different from either of the uninoculated groups and these three groups all showed significantly less bone loss than the other four. Thus, IV immunisation conferred complete protection against A. viscosus induced bone loss.

Tables 9.2 and 9.3 give the mean blastogenic response of MLN and spleen cells respectively, with and without I/E combined. One way analysis of variance showed that only with zero stimulation of both spleen and MLN cells, and PHA stimulation of MLN cells, was the variation between groups significantly greater than that within them. ($P < 0.05$).

The non-specific and specific anti-A. viscosus serum immunoglobulin concentrations are presented in Tables 9.4 and 9.5 respectively. One-way analysis of variance showed the variation between groups to be significantly greater than that within them for classes IgG and IgM in the non-specific investigations and classes IgG and IgA in the specific anti-A. viscosus assays. ($P < 0.05$).

Table 9.6 shows the ratio of specific/non-specific immunoglobulin concentration for each immunisation group in each of the three Ig classes. In class IgA the ratios associated with uninoculated controls and the IV group were lower than any of the others, but this pattern was not apparent in classes IgG and IgM.

Table 9.1 Bone loss (mm^2) 12 weeks after oral inoculation with A. viscosus, and statistical signficance of differences between groups.
n = number of mice. * $P < 0.05$

n	Mean ± s.e.		IV	Uninoc	Uninoc. (Main expt)	7-9	9-7	10^7	10^7 (main expt)
9	0.929 ± 0.008	IV							
10	0.932 ± 0.019	Uninoculated							
10	0.933 ± 0.030	Uninoculated (main expt)							
10	1.470 ± 0.010	7-9	*	*	*				
10	1.471 ± 0.010	9-7	*	*	*				
4	1.473 ± 0.006	10^7	*	*	*				
9	1.484 ± 0.024	10^7 (main expt)	*	*	*				

Table 9.2 Mean blastogenic response in cpm for MLN cells with and without I/E combined. Single cultures and co-cultures. n = number of repeat measurements.

Group		Stimulation		
		0	PHA	A. viscosus
Uninoculated (U)	n	41	24	22
	Mean	1061.9	21196.6	8837.5
	s.e.	126.6	2240.2	1541.7
10^7	n	41	24	24
	Mean	996.9	12669.1	8475.6
	s.e.	164.9	1747.9	1131.6
9-7	n	6	6	6
	Mean	1835.5	8475.2	7685.3
	s.e.	122.3	355.8	790.0
7-9	n	6	6	6
	Mean	2089.5	10458.8	10964.0
	s.e.	130.0	1375.2	1164.3
IV	n	12	5	6
	Mean	1187.0	12080.8	8504.8
	s.e.	155.0	470.7	432.4
$U/10^7$	n	3	3	3
	Mean	1876.3	5105.3	7465.0
	s.e.	66.3	1031.3	733.3
U/9-7	n	2	3	3
	Mean	1644.5	9055.3	7838.0
	s.e.	471.5	1000.4	357.4
U/7-9	n	3	3	3
	Mean	2086.7	9892.7	7876.7
	s.e.	191.4	1279.6	789.1
10^7/7-9	n	3	3	3
	Mean	2332.3	7083.0	8855.0
	s.e.	158.0	359.2	978.9
10^7/9-7	n	3	3	3
	Mean	3624.0	7143.3	9774.0
	s.e.	770.3	1020.4	747.6

Table 9.3 Mean blastogenic response in cpm for spleen cells with and without I/E combined. Single cultures and co-cultures. n = number of repeat measurements.

Group		Stimulation		
		0	PHA	A. viscosus
Uninoculated (U)	n	36	20	18
	Mean	2001.6	9298.4	10157.3
	s.e.	312.3	2159.1	1876.8
10^7	n	36	21	20
	Mean	2063.2	12262.6	7825.6
	s.e.	355.6	2851.7	1255.9
9-7	n	3	3	3
	Mean	3571.3	6261.7	6771.3
	s.e.	254.1	93.4	771.0
7-9	n	3	2	3
	Mean	4278.0	7888.5	14177.7
	s.e.	293.6	204.5	251.6
IV	n	11	6	6
	Mean	1122.4	18512.5	13056.3
	s.e.	246.7	1005.3	645.2
U/10^7	n	2	3	3
	Mean	5222.5	1676.7	3863.3
	s.e.	86.5	241.2	496.0
U/9-7	n	3	3	3
	Mean	4780.3	6357.3	7698.7
	s.e.	377.5	198.3	200.5
U/7-9	n	2	3	3
	Mean	6772.5	4752.3	7173.3
	s.e.	387.5	505.7	441.7
10^7/9-7	n	3	3	2
	Mean	6966.3	2452.0	3858.0
	s.e.	1780.7	91.8	695.0
10^7/7-9	n	3	3	3
	Mean	7220.0	1783.3	5750.7
	s.e.	751.4	291.6	299.7

Table 9.4 Non-specific immunoglobulin concentration by immunisation/inoculation group in mg μl^{-1} pooled serum.
n = number of repeat measurements made on pooled sample.

Group	IgG		IgA		IgM	
	n	Mean ± s.e.	n	Mean ± s.e.	n	Mean ± s.e.
Uninoculated	2	13.13 ± 1.94	2	176.67 ± 0.00	2	1.90 ± 0.38
10^7	2	113.38 ± 5.97	2	212.00 ± 8.83	2	10.64 ± 0.76
7-9	2	328.21 ± 5.97	2	207.59 ± 22.08	2	14.25 ± 0.95
9-7	2	328.21 ± 29.84	2	198.76 ± 4.42	2	14.63 ± 0.19
IV	2	202.89 ± 11.94	2	225.26 ± 4.42	2	11.78 ± 1.14

Table 9.5 Specific anti-*A. viscosus* immunoglobulin concentration by immunisation/inoculation group in mg ml^{-1} pooled serum for IgG and mg 100 ml^{-1} pooled serum for IgA and IgM. n = number of repeat measurements made on pooled sample.

	IgG		IgA		IgM	
Group	n	Mean ± s.e.	n	Mean ± s.e.	n	Mean ± s.e.
Uninoculated	6	4.10 ± 1.09	5	30.18 ± 3.81	6	4.08 ± 0.69
10^7	6	8.55 ± 1.39	5	283.59 ± 37.00	6	5.56 ± 0.69
7-9	3	98.66 ± 20.46	3	43.58 ± 22.47	3	8.32 ± 0.66
9-7	3	194.14 ± 39.88	3	47.52 ± 4.27	3	12.29 ± 5.27
IV	3	74.79 ± 7.84	2	32.39 ± 7.72	3	9.82 ± 1.14

Table 9.6 Ratio of specific/non-specific immunoglobulin concentration for each immunisation/inoculation group ($x10^6$).

Group	IgG	IgA	IgM
Uninoculated	312.3	1.7	21.5
10^7	75.4	13.4	5.2
7-9	300.6	2.1	5.8
9-7	591.5	2.4	8.4
IV	368.6	1.44	8.3

9.4 D I S C U S S I O N

The first two immunisation procedures, involving subcutaneous inoculation of 10^9 cfu heat-killed A. viscosus were used to introduce A. viscosus antigens to the animals without the danger of inducing disease or provoking a brisk acute inflammatory reaction. In group 9-7, it was thought that exposure to the antigens might reduce bone loss either by priming the immune system for an effective response to live organisms or, perhaps less likely, by inducing tolerance to the organism and so preventing a potentially damaging host response. The 7-9 group was included to see whether the proven destructive effect of the 10^7 cfu oral inoculations could be overcome by either an enhanced host immune response or tolerance subsequently induced by the subcutaneous injection. However, there were two potential difficulties in using subcutaneous inoculations of heat-killed organisms. First, it was not certain that heat-killed A. viscosus would retain all the antigenic specificities of their live counterparts, although this could not be confirmed without further investigation. Second, it could be argued that tolerance would more readily occur after oral rather than subcutaneous immunisation, although André et al (1975) showed that tolerance could be induced by intraperitoneal immunisation.

Intravenous immunisation was used to provide maximal exposure of the immune system to A. viscosus antigens. A possible difficulty here was the induction of generalised disease, but 9 of the original 10 mice in this group survived these inoculations without apparent distress. IV immunisation was the only procedure that was effective in protecting mice against periodontal damage from oral inoculations of live organisms (Table 9.1).

In terms of lymphocyte transformation, for both MLN and spleen cells, co-cultures tended to have higher values than their individual component cultures with zero stimulation but often lower values with PHA and A. viscosus. This implies mutual help and suppression in unstimulated and stimulated co-cultures respectively. Within stimulation groups, there was no obvious relationship of the findings with bone loss. All cultures reacted to stimulation, thus tolerance was apparently not induced.

In terms of immunoglobulin concentration, groups 7-9 and 9-7 tended to have relatively high values in classes IgG and IgM for both non-specific and specific anti-A. viscosus tests. This finding may have been caused by the fact that these immunisation procedures involved introduction of greater numbers of organisms, allbeit including some that had been heat-killed, than in any of the other groups. Also for classes IgG and IgM, IV inoculation of 10^5 cfu resulted in greater serum response, both non-specifically and specifically to A. viscosus, than did oral inoculations of 10^7 cfu. It must be assumed that the intravenous route resulted in greater antigen exposure to the immune system than the oral route, but the higher immunoglobulin level was not matched by higher bone loss.

The non-specific IgA data show no correlation with bone loss in that the uninoculated and IV groups, for which bone loss did not differ significantly, showed the lowest and highest serum concentrations respectively. For specific anti-A. viscosus IgA, however, the highest concentration was found in the 10^7 group and the lowest in the IV group and in uninoculated controls. Thus

it is only for specific anti-A. viscosus IgA that there was correlation with bone loss. This is consistent with the hypothesis that high levels of specific anti-A. viscosus IgA are associated with disease in the mouse model.

Again there was no indication of tolerance as all groups responded to stimulation with A. viscosus.

The authors of the original mouse model paper (Fitzgerald et al, 1981) have reported no immunisation experiments so no comparison of results is possible.

The conclusion from this brief study is that only IV immunisation was effective and that this induced the lowest serum specific anti-A. viscosus IgA response of the three immunisation attempts. The work could be continued by examining histological sections of the gingiva of immunised mice for the presence of IgA immune complexes in all the groups. It might be anticipated that only groups with high levels of bone loss would show these in any profusion. Further studies could also include salivary Ig analysis and the testing of alternative immunisation procedures.

CHAPTER 10

OVERVIEW

10.1 THE PRESENT STUDY

Two problems are encountered in the study of periodontal disease. First, the weight of evidence suggests that the natural history and progression of the disease is essentially cyclic in nature (Hirschfeld & Wasserman, 1978; Becker et al, 1979; Socransky, 1984). Examples exist which show that active periodontitis may undergo spontaneous arrest and that, in any given periodontitis patient at any one time, only some sites have active disease (Moskow, 1978; Vandesteen et al, 1981). This means that clinical and laboratory findings may not correlate with disease activity. Second, many body systems are involved in the pathogenesis of periodontal disease and it is not easy to assess the contribution of each of these, alone or through interaction with others. The A. viscosus mouse model, however, appears to be a way of circumventing these problems, in that it provides a means of inducing periodontal disease in a reproducible but adaptable manner.

Periodontal research has provided three principles relevant to the present study:-

(a) All mammals from mice to men have only a limited number of defence responses to microbial challenge or tissue injury. These self-protection mechanisms, when examined in isolation, react in a consistent and predictable manner and vary little between species.

(b) The host defence system also participates in host tissue damage, thus activation of any one of its constituent parts, be it the immune response, acute inflammation, complement cascade,

phagocytic leucocytes or various combinations of these, brings to bear the apparently opposing forces of protection and destruction. In most cases, the price of damage paid is very small relative to the protection gained.

(c) The factors involved in determining whether and to what extent host damage occurs and how long the destructive process continues are only poorly understood.

In no known case is periodontitis in animals identical to that seen in humans, so no true analogue of the human disease has been found. At first sight this appears to be a tremendous handicap to periodontal disease research. However, the different animals available, each with their own special features, provide many investigative opportunities. Nevertheless, the questions asked of each animal model must be precisely defined and the animal chosen with great care. For investigations of microbially induced alveolar bone destruction, rodents are particularly useful.

Mice are very small relative to humans and their periodontal structures are minute. On the other hand, the infecting bacteria and both resident cells and infiltrating leucocytes are of roughly the same dimensions in mice and men. Mice live for only 1-2 years, thus the rates of growth and development are relatively high causing rapid shifts in the relationships between teeth and jaws. In man, tissue turnover is much slower. The opportunities for microbial interference are therefore very different in these two species. Of relevance to this difference is the work of Garant & Cho (1979) in rats, which demonstrated that bone

resorption occurs up to a finite distance away from the plaque causing the loss. Waerhaug (1979a & b) applied this principle to human periodontitis, and this helps explain why mice, with their thin bone, suffer much greater bone loss relative to their size than do human periodontitis patients.

The current work was an attempt to shed light on the functions and actions of three different major components of the host defence system, phagocytic cells (Chapter 6), the cellular immune response (Chapter 7) and the humoral immune response (Chapter 8). The most important finding was the peak of specific anti-A. viscosus IgA found in the serum of animals orally inoculated with 10^7 cfu live A. viscosus, consistently in all genotypes examined and associated with maximum bone loss. The hypothesis that low and high dose tolerance operated in the 10^6 cfu and 10^9 cfu inoculated animals is attractive and, while confirmation of this is not possible with the present results, there is certainly no indication to the contrary.

In theory, any disease of microbial origin could be prevented by immunising the susceptible host at the appropriate time with a vaccine containing the necessary protective antigen or antigens. Many diseases previously responsible for much mortality and morbidity have now been greatly reduced in incidence or eliminated by vaccination. The presence of antibody per se is merely an indication of antigenic exposure and whether or not an antibody is protective or not has to be established. If the antibody is found to be protective, then immunisation would be protective. If the antibody is found to be damaging, then

enhancement would accelerate the disease. In the latter case, which appears to apply to specific anti-A. viscosus serum IgA, immunisation, if it were to be protective, would have to be directed towards desensitisation or the induction of specific immune tolerance. Is it possible that this occurred in the 10^6 cfu and 10^9 inoculated mice?

Perhaps the next effective approach to immunisation in the A. viscosus model would be the induction of antibodies to inhibit colonisation of the organisms, which would presumably render arguments about protective or damaging immune responses irrelevant. It is in this respect that the recent work of Donkersloot et al (1985), Clark et al (1984) and Clark (1985), which investigated the role of fimbriae on the colonisation of tooth surfaces by A. viscosus, may prove to be important.

The present work has demonstrated a tantalising association between bone loss and specific IgA concentration, but has also posed many additional questions to those it set out to answer. Various possibilities for further studies are outlined below and it is hoped that these, if implemented, might help to shed light on some of these questions.

10.2 POSSIBILITIES FOR FUTURE WORK

There are attractions in extending the work to see whether similar results to those presented here could be obtained using oral inoculations of other periodontopathic bacteria such as Actinobacillus actinomycetemcomitans, Capnocytophaga species or Bacteroides gingivalis in place of A. viscosus. Other possibilities include increasing the number of mouse genotypes subjected to the A. viscosus inoculation schedule, or adding to the existing information by examining the effects of 10^8 cfu A. viscosus inoculations in the genotypes already studied. Perhaps the most interesting avenue to explore, however, would be attempting to increase our understanding of what is happening in the existing A. viscosus inoculation model in BALB/c mice. Various possibilities come to mind:-

1. Try to correlate the histological picture with bone loss. It would be valuable to perform weekly sacrifices and subsequent serial sectioning of both uninoculated and 10^7 cfu inoculated BALB/c mice. This is currently in progress.

2. Attempt separation, isolation and purification of the A. viscosus EDTA extract into its constituent fragments with the intention of identifying the antigenic/mitogenic constituents. This would be a cornerstone of all further immunological work. Such specific agent(s) could then be added to lymphocyte cultures, chemiluminescence assays and ELISA plates, thus increasing the accuracy of such experimentation. Obtaining these fragment(s) would be the result of a protracted period of specialised work, which would require a great deal of detailed bacteriological

knowledge. It would therefore be logical to embark on a joint venture in collaboration with a microbiologist.

3. Separate lymphocytes for the cell-mediated immunity studies into the constituent B and T cell subsets. If they were available, the antigenic/mitogenic A. viscosus standard fragment(s) could be used in addition to the standard mitogens to stimulate cultures of separated B and T cells.

4. Continue humoral immunity studies. The major area of extension here would be to measure the salivary levels of anti-A. viscosus antibody found in the different inoculation groups. It is anticipated that this would be possible employing the ELISA technique and the isolated standard A. viscosus antigenic/mitogenic fragment(s). Should greater accuracy be required, however, gel electrophoresis could be used.

5. Continue the PMN work using both MPO and chemiluminescence assays for both uninoculated and 10^7 cfu inoculated BALB/c mice. In addition, the chemiluminescence assays could have either whole A. viscosus organisms or the standard antigenic/mitogenic fragment(s) thereof as stimulants.

APPENDIX

All chemicals in this section were obtained from B.D.H. (Poole, UK) unless stated otherwise.

Acetate Buffer

5.75 ml	glacial acetic acid
94.25 ml	distilled water

Take 20 ml of this and add to 6 ml of 1 M NaOH.
Make this up to 100 ml with distilled water. pH = 4.2

Coating Buffer

1.59 g	sodium carbonate
2.93 g	sodium hydrogen carbonate
0.2 g	sodium azide

Make up to 1 litre.

Cooked Meat Broth (CMB)

25 g	basic nutrient broth (Oxoid Ltd, Basingstoke, UK)
1 litre	distilled water

Add 10 ml to a 1 inch vial with $\frac{1}{4}$ inch cooked meat.

Diet

(i) Normal diet

Oxoid mouse diet (Oxoid Ltd, Basingstoke, UK)

(ii) Special diet

40 g	sucrose
15 g	wheat flour
32 g	skimmed milk
5 g	brewers' yeast
2 g	vitamin-mineral protein supplement

Formic Citrate

35 ml	formic acid
65 ml	20% sodium citrate

Nitrate Test: Solution 1

0.5 g	sulphanilic acid
30 ml	glacial acetic acid
120 ml	distilled water

Nitrate Test: Solution 2

0.2 g	1,6 cleves acid
30 ml	glacial acetic acid
120 ml	distilled water

PBS Tween

8 g	sodium chloride
0.2 g	potassium dihydrogen orthophosphate
2.9 g	disodium hydrogen orthophosphate dodecahydrate
0.2 g	potassium chloride
0.5 ml	Tween 20

Make up to 1 litre.

Phosphate Substrate Solution

1 phosphatase substrate tablet in 5 ml of diethanolamine.
(Sigma Chemical Co Ltd, Poole, UK)

Diethanolamine

97 ml	diethanolamine
800 ml	distilled water
0.2 g	$NaCo_3$
100 mg	$MgCl_2$ $6H_2O$
1 M	HCR - pH 9.8

Make up to 1 litre with distilled water.

Proteose Peptone Yeast Medium (PPY)

2 g	proteose peptone (Oxoid Ltd, Basingstoke, UK)
1 g	yeast extract (Difco Laboratories, East Molesey, UK)
0.5 g	NaCl
100 ml	distilled water
2 ml	3.75% cysteine hydrochloride
2 ml	2% Na_2CO_3
2 ml	haemin (250 ug/ml)
2 ml	menodione (50 ug/ml)

Staining procedures

Azure

1. Sections to water.
2. Azure stain - 10 mins.
3. Directly into absolute alcohol to dehydrate.
4. Clear and mount.

Azure stain

0.1% in AzurA in 30% alchohol

H + E

1. Sections to water.
2. Mayer's haematoxylin - 3 mins.
3. Wash in water.
4. Saturated lithium carbonate - 1 min (to blue haematoxylin).
5. Wash in water.
6. 1% eosin in 1% calcium chloride - 3 mins.
7. Wash in water.
8. Dehydrate, clear and mount.

Van Gieson

1. Sections to water.
2. Celestin blue/haemalum - 5 mins each.
3. Wash well in water.
4. Van Gieson stain - 5 mins.
5. Rinse in distilled water.
6. Dehydrate, clear and mount.

Van Gieson stain

100 ml	saturated aqueous picric acid
5-10 ml	1% aqueous acid fuchsin

Standard solutions for gas liquid chromatography

volatile fatty acids

0.01 M	acetic
0.01 M	propionic
0.01 M	iso-butyric
0.01 M	n-butyric
0.01 M	iso-valeric
0.01 M	n-valeric
0.01 M	iso-caproic
0.01 M	n-caproic

non-volatile

0.04 M	lactic
0.01 M	succinic

Store at 4°C.

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